# CANADIAN JOURNAL OF RESEARCH

VOLUME 28 APRIL, 1950 NUMBER 2

— SECTION C —

# BOTANICAL SCIENCES

## *Contents*

Page

The Sterilization of Glass and Calomel Electrodes by Ultraviolet Irradiation—*R. W. Watson, Mary T. Clement, and D. R. Muirhead* - - - - - - - - - - - - - 183

Influence of the Amino Acid-Dextrose Reaction on Growth of *Rhizobium*—*Dyson Rose and Ruth Peterson* - - - - - - 197

The Intermediate Metabolism of *Pseudomonas aeruginosa*. IV. The Absence of an Embden-Meyerhof System as Evidenced by Phosphorus Distribution—*Jack J. R. Campbell and Flora C. Norris* - - - - - - - - - - - - - 203

Biochemistry of the Ustilaginales. I. Preliminary Cultural Studies of *Ustilago zeae*—*R. H. Haskins* - - - - - - 213

NATIONAL RESEARCH COUNCIL
OTTAWA, CANADA

# CANADIAN JOURNAL OF RESEARCH

The *Canadian Journal of Research* is issued in six sections, as follows:

| | |
|---|---|
| A. Physical Sciences | D. Zoological Sciences |
| B. Chemical Sciences | E. Medical Sciences |
| C. Botanical Sciences | F. Technology |

For the present, Sections A, C, D, and E are to be issued six times annually, and Sections B and F, twelve times annually, each under separate cover, with separate pagination.

The *Canadian Journal of Research* is published by the National Research Council of Canada under authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. The *Canadian Journal of Research* is edited by a joint Editorial Board consisting of members of the National Research Council of Canada, the Royal Society of Canada, and the Chemical Institute of Canada.

Sections B and F of the *Canadian Journal of Research* have been chosen by the Chemical Institute of Canada as its medium of publication for scientific papers.

## EDITORIAL BOARD

*Representing*
NATIONAL RESEARCH COUNCIL

DR. H. P. ARMES (*Chairman*),
Dean of the University,
University of Manitoba,
Winnipeg, Man.

* DR. G. H. HENDERSON,
Professor of Mathematical Physics,
Dalhousie University, Halifax.

DR. ROBERT NEWTON,
President, University of Alberta,
Edmonton, Alta.

DR. C. H. BEST,
The Banting and Best Department of Medical Research,
University of Toronto, Toronto.

*Representing*
ROYAL SOCIETY OF CANADA

DR. A. NORMAN SHAW,
Chairman, Department of Physics,
McGill University, Montreal.

DR. J. W. T. SPINKS,
Head, Department of Chemistry,
University of Saskatchewan,
Saskatoon.

} Section III

DR. H. S. JACKSON,
Head, Department of Botany,
University of Toronto, Toronto.

DR. E. HORNE CRAIGIE,
Department of Zoology,
University of Toronto,
Toronto.

} Section V

*Ex officio*

DR. LÉO MARION, Editor-in-Chief,
Division of Chemistry,
National Research Laboratories,
Ottawa.

DR. H. H. SAUNDERSON,
Director, Division of Information Services,
National Research Council,
Ottawa.

*Representing*
THE CHEMICAL INSTITUTE OF CANADA

DR. H. G. THODE,
Department of Chemistry,
McMaster University,
Hamilton.

## EDITORIAL COMMITTEE

Editor-in-Chief, DR. LÉO MARION
Editor, Section A, DR. A. NORMAN SHAW
Editor, Section B, DR. J. W. T. SPINKS, DR. H. G. THODE
Editor, Section C, DR. H. S. JACKSON
Editor, Section D, DR. E. HORNE CRAIGIE
Editor, Section E, DR. J. B. COLLIP
Editor, Section F, DR. J. A. ANDERSON, DR. A. NORMAN SHAW, DR. H. G. THODE

Manuscripts should be addressed:

*Editor-in-Chief,*
*Canadian Journal of Research,*
*National Research Council, Ottawa, Canada.*

* *Deceased.*

# Canadian Journal of Research

*Issued by* THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 28, SEC. C. APRIL, 1950 NUMBER 2


## THE STERILIZATION OF GLASS AND CALOMEL ELECTRODES BY ULTRAVIOLET IRRADIATION[1]


BY R. W. WATSON,[2] MARY T. CLEMENT,[3] AND D. R. MUIRHEAD[4]



### Abstract

By strictly defining the procedure for handling and storing the electrodes, contamination was limited to microorganisms from the air. For the purpose of this investigation, therefore, sterilization is defined as the complete inactivation or killing of air-borne bacteria, yeasts, and molds. If the organisms are not present in such a way as to offer mutual protection from the radiation, the total energy at 2537 Å necessary to inactivate the most resistant organism may be assumed to ensure sterilization. *Aspergillus niger* v. Tiegh. was identified as the most resistant contaminant, and electrostatic sampling showed that clumps containing up to about 40 spores of *A. niger* Strain 6277 became air-borne. These clumps required approximately 60,000 μw. min. per cm.$^2$ at 2537 Å for inactivation. Used with the accessory procedures, exposure to 680 μw. per cm.$^2$ for two hours was found to provide a safe margin and to ensure sterilization of carefully cleaned and specially mounted electrodes. A satisfactory design for a crevice-free glass electrode sterilizable by ultraviolet light is described. E.m.f. changes in glass–calomel cells sterilized by the recommended procedure do not exceed 0.4 mv., corresponding to 0.006 pH units.


### Introduction

The maintenance of cultural purity in pH-controlled fermentation media is primarily dependent on a method for sterilizing the electrodes without altering their calibration. All other necessary equipment and materials may be steam sterilized, but several authors have noted that glass electrodes lose their calibration when autoclaved (4, 14, 15). To overcome this difficulty Longsworth and MacInnes (14) proposed a chemical sterilization with 0.1% mercuric chloride. The same authors later described an apparatus in which the electrodes were chemically sterilized, rinsed, and inserted into the medium without coming in contact with laboratory air (15). Neish and Ledingham (22) in their study of 2,3-butanediol fermentations at poised hydrogen ion concentrations described a useful ultraviolet sterilizer for electrodes, and outlined a preliminary procedure. From a survey of the physical methods of sterilization (25), irradiation at 2537 Å offers several advantages. It is easy to use, has a rapid bactericidal action (16, 18), eliminates the need for rinsing (15), and avoids the introduction of trace or toxic substances into the medium.


[1] *Manuscript received December 27, 1949.*
*Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as Paper No. 81 on the Industrial Utilization of Wastes and Surpluses, and as N.R.C. No. 2090.*



[2] *Biochemist, Industrial Utilization Investigations.*
[3] *Microbiologist, Industrial Utilization Investigations.*
[4] *Technical Officer, Industrial Utilization Investigations.*



[The February issue of Section C (Can. J. Research, C, 28:1-182. 1950) was issued March 29, 1950.]

From the absence of any reported deterioration of electrode glass by ultraviolet light *per se*, irradiation may be expected to have little or no effect on calibration. The problem of electrode sterilization is obviously of critical importance in the application of precision pH control to fermentation media (14, 15, 22, 27), and the present communication reports an analysis of the significant factors in sterilization procedure.

A method was first outlined for handling and storing the electrodes so that contamination might be restricted to microorganisms from the air. For the purpose of this investigation, therefore, sterilization is defined as the complete inactivation or killing of air-borne bacteria, yeasts, and molds. So far as is known the conditions necessary for electrode sterilization by irradiation have not previously been established. Koller (11) made pertinent observations on the sterilization of glassware by irradiation, and noted the large increase in total energy which must be applied for inactivation when organisms are protected by even a thin film of grease. The sterilization of glass electrodes by ultraviolet light presents a different problem from that of other glassware, in that desiccation of certain types of electrode glass may cause subsequent variations in potential, and damage to the superficial silica gel membranes of the bulbs (4, 7, 12, 20). Immersion of the electrodes in water during the period of irradiation not only prevents desiccation, but improves the conditions for sterilization.

Many investigators have determined the total energies in absolute units at 2537 Å necessary to suppress the development of bacteria, yeasts, and molds. Assuming that the total energy necessary to inactivate the most resistant organism ensures sterilization, it becomes necessary to identify the most resistant contaminant. This assumption is valid only so long as the organisms are not present in sufficient concentration or in such a way as to afford mutual protection from the radiation.

All authors agree that low concentrations of the vegetative cells of bacteria may be easily killed by irradiation at 2537 Å. Different species of bacteria differ relatively little in their sensitivity to ultraviolet light. The lower and upper limits among 12 species listed by Nagy (21) are 103 $\mu$w. min. per cm.$^2$ for *Serratia marcescens*, and 1247 $\mu$w. min. per cm.$^2$ for *Proteus vulgaris*. Rahn (25) quotes a suggestion by Wyckoff that differences in the sensitivity of different species are correlated with size. The careful work of Luckiesh and Holladay (16) and Luckiesh and Knowles (18) has shown that dilute suspensions of *Escherichia coli* in clear water are inactivated by 120 $\mu$w. min. per cm.$^2$ This confirms a previous estimate by Koller (11). By collating the results of many authors, Hollaender (8, 9) showed that deviations of about 300% occur in estimates of the energy in absolute units necessary to ensure 90% inactivation of *E. coli* in liquid suspension or on an agar surface. These variations may be explained by assuming that organisms are more sensitive at certain stages in the life cycle (8).

When measurements in air and water are compared a greater discrepancy appears. Wells and Fair (28) state that the lethal effect of ultraviolet light

in dry air is from 50 to 100 times greater than when the organisms are exposed on the surface of Petri plates, or are irradiated in water. This general finding has been confirmed by Koller (11), Luckiesh and Holladay (16), and others, who conclude that bacteria in water, or in air of high relative humidity require about 10 times more energy to suppress their development than when suspended in dry air. Rentschler and Nagy (26), however, hold the view that the resistance of bacteria to irradiation is the same, whether in water or in air, and ascribe the differences cited to sampling errors.

Even the development of bacterial spores is readily inhibited. Nagy (21) gives a lower estimate (132 $\mu$w. min. per cm.$^2$) for the energy necessary to kill the spores of *Bacillus subtilis* than for that to inactivate the vegetative cells (183 $\mu$w. min. per cm.$^2$). Duggar and Hollaender (5) found that the vegetative cells of *B. subtilis* and *B. megatherium* required slightly less energy than the spores for 85% killing. The spores of *B. mesentericus* have been found to require about five times as much energy for inactivation as *E. coli* (13). Other reports confirm these observations, and show that there is little difference between the sensitivity of bacterial spores and of vegetative cells to ultraviolet light.

The resistance of yeast cells has been reported to be comparable to that of bacteria. According to Nagy (21) the lower and upper limits of the energy necessary to inactivate six types of yeast range from 110 to 280 $\mu$w. min. per cm.$^2$ Luyet (19) found that the development of yeast cells was inhibited by about one-sixth of the energy necessary to destroy spores of the mold, *Rhizopus nigricans*. In absolute units this becomes about 600 $\mu$w. min. per cm.$^2$, or slightly more than the energy necessary to inactivate *Sarcina*, and about half of that required by *E. coli* (21).

The higher resistance of mold spores has been correlated with the increased size of the cells, and the thickness and pigmentation of the walls (25). The total energy necessary to inhibit the growth of single spores of many common molds may be estimated from the data of Fulton and Coblentz (6), Koller (11), Conklin (3), and Nagy (21). Identification of the most resistant local airborne fungi therefore became of first importance in an attempt to sterilize glass surfaces by ultraviolet irradiation.

## Experimental

The microorganisms in known volumes of the air of the laboratory and of adjacent rooms and corridors were precipitated electrostatically on tryptose–blood agar in Petri dishes by means of a Luckiesh–Holladay–Taylor sampler (17). After incubation at 30° C. the fungi which had developed were identified.

The effectiveness of overnight irradiation of the air of the laboratory (10 ft. × 12 ft. × 15 ft.) with three suitably mounted 30 w. germicidal lamps was estimated by sampling at intervals before and after the irradiation period. These tests revealed the prevalence of *Aspergillus niger* as an air-contaminant, and available data indicated that this species is probably the most resistant local organism (*vide infra*).

To determine the energy necessary to inactivate spores of *A. niger*, a sterilizer of anodized aluminium with three 15 w. G.E. germicidal lamps at the focus of parabolic reflectors, as designed by Neish and Ledingham (22), was used. This design provided cross-fire irradiation of objects between the lateral lamps. Six 8 in. × 1 in. test tubes of special 254 UV transmission glass (Pyrex 9741) were suspended through holes drilled in the aluminium top. Their ends were approximately 6 in., 6 in., and 2 in. from the three lamps. At the time of each test the transmission at 2537 Å of the glass in the test tubes was measured with a Beckman DUV spectrophotometer. The intensity of the energy at 2537 Å at the six faces of an imaginary cube in the position of the lower ends of the test tubes as measured with a Weston Model BS 1205 ultraviolet meter was as follows: at the four vertical faces of the cube, 900, 1580, 1050, and 1650 $\mu$w. per cm.$^2$, and at the upper and lower faces 680 and 2400 $\mu$w. per cm.$^2$ respectively.

The following method was used to estimate the energy at 2537 Å necessary to kill single spores of *A. niger* ATCC Strain 6277. The spores were removed from a 14-day agar slant by scraping the surface, and transferred to 500 ml. of sterile water. The suspension was filtered aseptically through successive layers of absorbent cotton, and examined microscopically for clumping. After filtration through three layers almost all the spores were present singly, although occasional pairs and a few larger clumps containing a maximum of six spores were found. The number of spores per ml. (ca. 29,000) was estimated from haemocytometer counts. To each of the experimental test tubes, 25 ml. of the suspension, containing approximately 725,000 spores, was added aseptically. Duplicate pairs were irradiated in the sterilizer for 4, 8, 10, 20, 40, and 60 min., the tubes being removed and shaken at 10-min. intervals. The temperature in the tubes rose to 45° to 50° C. in the first five minutes, and then remained fairly constant. After irradiation, the spores were again uniformly distributed by shaking, and 0.5 ml. from each tube was pipetted aseptically to an agar plate. The spores were streaked as evenly as possible over the surface with a sterile needle, and the plates incubated at 30° C.

Previous authors have determined the energy at 2537 Å necessary to inactivate only single spores of *A. niger*. When clumping is present the spores toward the center of the cluster tend to be shaded from the radiation and an increased total energy is required to suppress their development. A method was therefore devised to determine whether *A. niger* is disseminated by air as single spores or whether spore dispersal by air-borne clumps also occurs. A closed room was contaminated by tapping an open agar plate containing an 18-day culture of Strain 6277 in a current of air, and the room air was kept in circulation by an electric fan for five minutes before sampling. The spores were collected by means of the Luckiesh–Holladay–Taylor electrostatic sampler (17), using water in place of agar. Microscopic examination of the suspension showed an abundance of both single spores and spore clumps. The largest clumps contained about 40 spores. The velocity of settling of the larger clumps in water suggests that they probably settle rapidly in still air.

To determine the energy necessary to inactivate air-borne spore clumps of *A. niger* on an agar surface, a 15 w. germicidal lamp in series with a constant voltage transformer was mounted horizontally above an outlined semicircular area half the size of a Petri dish. The height of the lamp was adjusted until the incident intensity in the plane of an agar surface over the outlined semicircular area was 1022 $\mu$w. per cm.$^2$ This value represents an average of 11 readings with the Weston meter. The readings showed a deviation of $\pm 3\%$ over the area and duplicate series checked within $\pm 0.3\%$ if the lamp were left on about 15 min. before the first reading. Air-borne spores and spore clumps of *A. niger* Strain 6277 were obtained for irradiation from the contaminated air of the previously described closed room by exposure of agar plates for three minutes in the electrostatic sampler. The agar plates from the positively charged electrode of the sampler were kept as controls: those from the negative electrode were irradiated for 5, 10, 15, 20, 30, 40, and 60 min. at 1022 $\mu$w. per cm.$^2$ To allow for delayed development following irradiation the plates were kept under observation for seven days. Only the colonies that developed over the semicircular area irradiated with a measured uniform energy were counted. A series of 10 pairs of plates was also taken and incubated at 30° C. for counts of the relative number of spores deposited over the positive and negative electrodes.

The argument that the total energy necessary to ensure sterilization is equal to that necessary to inactivate the largest air-borne spore clumps of *A. niger* depends on (1) prevention of the development of more resistant colonies of microorganisms on electrodes during storage and (2) elimination of other than air-borne contaminants. It was observed that the glass stems of electrodes stored in unsterilized distilled water became coated with bacterial slime. Above the water line in the standard taper (24/40), 500 ml. Erlenmeyer flasks used to house the electrodes, colonies of sporulating molds also developed on the electrode stems. Even careful cleaning failed to remove such growths completely, and submacroscopic particles or islands of microorganisms remained adhering to the glass. To obtain an estimate of the resistance of colonies formed in storage to irradiation, two electrodes so contaminated were exposed for five hours in the sterilizer. During this period each electrode received from 200,000 to 300,000 $\mu$w. min. per cm.$^2$ After irradiation the electrodes were transferred through sterile air to sterile nutrient media, and incubated for 24 hr. at 30° C. To estimate the energy necessary to inactivate bacteria alone in small visible particles, the organisms from a 48 hr. agar slant of *Bacillus polymyxa* Strain C42(3)E13 were transferred to sterile distilled water. The gummy polysaccharide forming the outer wall of these bacteria tends to prevent separation of the cells. Several irregular clumps less than 1 mm. in diameter were transferred in 5 ml. of water to each of three test tubes, and irradiated in the sterilizer for one, two, and three hours respectively. Five milliliters of sterile broth was then added, and the media incubated. The transmission of each test tube at 2537 Å was measured after the experiment with a Beckman DUV spectrophotometer.

Koller (11) demonstrated that only crevice-free glass surfaces may be sterilized by ultraviolet irradiation. Therefore, attempts were made to mount commercial glass electrodes (e.g. Leeds and Northrup Standard 1199-32 with 7 in. glass buffer bridges) in reduced 24/40 tapers in such a way as to have the seals crevice-free. For this purpose rubber, wax, and glyptal and polystyrene cements were used. Electrodes so sealed were exposed to 1000 $\mu$w. per cm.$^2$ at 2537 Å for one hour, and tested under the conditions described for fermentations at controlled pH (22, 27). The test organisms included *Aerobacter aerogenes*, *S. marcescens*, and *B. polymyxa*. Each inoculum was examined microscopically for purity, and at the end of the fermentation period, the media were plated and examined as Gram-stained smears.

Calomel electrode assemblies invariably contain crevices at the liquid junctions. Inspection of several commercial types showed that the special calomel electrodes recommended by Neish and Ledingham (22) were preferable. These may be simply made (22) by joining the end of a Leeds and Northrup 1199-27A salt bridge tube to a reduced 24/40 taper, filling the bridge with saturated aqueous potassium chloride and inserting the Leeds and Northrup Standard 1199-25A calomel electrode in the top as shown in Fig. 1. The sterilizability of two of these electrodes by irradiation was tested. After immersion for 24 hr. in a heavy suspension of vegetative cells of *B. polymyxa* Strain C42(3)E13 the electrodes were irradiated for 60 min. at an average of 1325 $\mu$w. per cm.$^2$, and transferred aseptically to tubes of nutrient broth for incubation at 32° C.

Once the optimum total energy for sterilization (1000 $\mu$w. per cm.$^2$ at 2537 Å for 60 min.) had been established, both the effectiveness of this energy in killing local air-borne contaminants and its effect on previous electrode calibrations were tested. Approximately 1400 cu. ft. of laboratory air was drawn over a period of six days through two Pyrex ultrafilters covered with sterile washed 60 to 80 mesh Pyrex particles. The collected microorganisms were transferred to sterile water, and 5 ml. of the suspension added to each of 16 quartz test tubes. The tubes were then irradiated for one hour in the sterilizer except for four tubes that were left as unirradiated controls. Five milliliters of sterile nutrient broth was then added aseptically to each tube, and the cultures incubated for seven days at 30° C. In irradiation tests of previously calibrated glass-calomel cells, the glass electrode bulbs were immersed in distilled water in dishes of Vycor 791 (23). Both electrodes were then exposed to 680 $\mu$w. per cm.$^2$ for two hours and the cells recalibrated on a precision pH monitor (1, 2).

## Results

Identification of the fungi trapped by a Luckiesh–Holladay–Taylor sampler from the air of the laboratory and from five adjacent rooms and corridors showed the presence of *Aspergillus niger* v. Tiegh., *A. flavus* Link., *A. ochraceus* Wilhelm, *A. ustus* (Bainier) Thom and Church, *A. versicolor* (Vuill.) Tiraboschi (?), *Cladosporium herbarum* Link., *Rhizopus nigricans*

Ehrb., *Trichoderma viride* Persoon, and species of *Penicillium*, *Cephalosporium*, *Fusarium*, *Neurospora*, *Phoma*, and *Scopulariopsis*. The total energy (Et) at 2537 Å necessary to suppress the development of spores of *A. niger* is probably greater than that for any of the other identified organisms (3, 6, 21).

Concentrations of the spores of *A. niger* in the laboratory air varied unpredictably. In Table I counts of bacteria + yeast and of mold colonies

TABLE I

CONTROL OF AIR-BORNE ORGANISMS BY IRRADIATION AT 2537 Å

| Time | Colonies present after 48 hr. at 30° C. | | |
|---|---|---|---|
| | Bacteria + yeast | Mold | Identity |
| 1620 | 40 | 1 | |
| 1630 | 46 | 4 | |
| *Intervening irradiation period 1630 – 0830 = 16 hr.* | | | |
| 0830 | 3 | 1 | |
| 0840 | 4 | 0 | |
| 0900 | 8 | 0 | |
| 0920 | 13 | 0 | |
| 1410 | 26 | 0 | |
| 1600 | 26 | 2 | *Penicillium* sp. |
| 1610 | 9 | 83 | *Aspergillus niger* Strain 215 |
| *Intervening irradiation period 1630 – 0830 = 16 hr.* | | | |
| 0840 | 9 | 2 | |

before and after irradiation of the room for 16-hr. periods are given. The number of colonies in each count represents the sum from the positive and negative plates of the sampler. The sampling time for each count was 10 min., and the volume of air passing the charged plates was approximately 5 cu. ft. (17). After irradiation of the closed room overnight the number of microorganisms trapped in a 10 min. period had decreased by about 90%. In this decrease are included both those organisms inactivated or killed by the radiation and those that had been removed from the air by settling. Contamination gradually increased during the day. The sudden increase in the number of *A. niger* spores of a strain different from the organisms used in these experiments (Table I) may have been due to the entrance of a contaminated air current or possibly to the presence of a worker from rooms where investigations with Strain 215 were in progress.

The approximate energy at 2537 Å necessary to kill suspensions of single spores of *A. niger* Strain 6277 in water may be estimated from Table II. The difficulty of eliminating spore clumps from the suspension was noted previously.

TABLE II

ENERGY AT 2537 Å NECESSARY TO KILL SINGLE SPORES OF *A. niger* STRAIN 6277 IN WATER

| Irradiation period, min. | Total energy, μw. min./cm.² | No. of spores per plate | No. of colonies after: | | | Survival ratio |
|---|---|---|---|---|---|---|
| | | | 2 days | 4 days | 10 days | |
| 4 | 3750 | 14,500 | 17 | 19 | | 0.001 |
| 8 | 7500 | 14,500 | 0 | 0 | 0 | $<1 \times 10^{-4}$ |
| 10 | 11,150 | 14,500 | 4 | 6 | | 0.004 |
| 20 | 22,300 | 14,500 | 0 | 0 | 0 | $<1 \times 10^{-4}$ |
| 40 | 44,600 | 14,500 | 0 | 0 | 0 | $<1 \times 10^{-4}$ |
| 60 | 66,900 | 14,500 | 0 | 0 | 0 | $<1 \times 10^{-4}$ |

The six colonies that developed after irradiation with 11,150 μw. min. per cm.² were probably from protected spores toward the center of small clumps. The data indicate that <7500μw. min. per cm.² is sufficient to reduce the survival ratio of single spores suspended in water to $<1 \times 10^{-4}$.

The lethal energy from G.E. germicidal lamps necessary to inhibit the development of spores and spore clumps of the same strain (6277) trapped electrostatically from the air of a contaminated room is shown in Table III.

TABLE III

TOTAL ENERGY (Et) AT 2537 Å NECESSARY TO INACTIVATE AIR-BORNE SPORE CLUMPS OF *A. niger* Strain 6277

| Irradiation period, min. | Average intensity, μw./cm.² | Total energy (Et) μw. min./cm.² | Colonies surviving after incubation for: | | | Colonies on control |
|---|---|---|---|---|---|---|
| | | | 2 days | 3 days | 7 days | |
| 5 | 1022 | 5110 | 28 | | | 125 |
| 10 | 1022 | 10,220 | 12 | | | 148 |
| 15 | 1022 | 15,330 | 5 | 8 | | 83 |
| 20 | 1022 | 20,440 | 7 | 7 | | 105 |
| 30 | 1022 | 30,660 | 0 | 1 | | 65 |
| 40 | 1022 | 40,880 | 2 | 3 | | 105 |
| 60 | 1022 | 61,320 | 0 | 0 | 0 | 72 |

Under the stated conditions 61,320 μw. min. per cm.² apparently ensures the inactivation of the spore clumps trapped from the air. Averaging the counts from 10 pairs of plates showed that 5% more colonies developed on plates from the negative electrode.

Colonies of sporulating molds, developed on electrode stems during storage, after irradiation for five hours with 200,000 to 300,000 μw. min. per cm.² and subsequent transfer to sterile media showed a rapid renewal of growth. After 24 hr. at 30° C. the broth media showed a dense growth of bacteria, and also contained numerous small mold colonies. The three test tubes containing

visible clumps of *B. polymyxa* in sterile distilled water, irradiated with approximately 39,000, 78,000, and 107,000 μw. min. per cm.² respectively, showed inactivation of all cells only after exposure to 107,000 μw. min. per cm.² After incubation for seven days at 30° C. there was no evidence of renewed growth in this tube. After incubation in sterile broth for 24 hr. at 30° C., the remaining tubes receiving 39,000 and 78,000 μw. min. per cm.² showed survival and growth.

Standard commercial electrodes sealed through reduced 24/40 tapers as described in the experimental section proved to be unsterilizable by ultraviolet irradiation. The organisms utilized in fermentations at controlled pH, viz. *A. aerogenes*, *S. marcescens*, and *B. polymyxa* survived in crevices that developed or were present in the various types of sealing material. In this way electrodes used to control pH in pure cultures of *S. marcescens*, and subsequently exposed to 60,000 μw. min. per cm.² at 2537 Å carried a sufficiently large number of viable organisms to provide heavy contamination in pure cultures of *B. polymyxa*. It was concluded in conformity with Koller (11) that only glassware with completely crevice-free surfaces was sterilizable by ultraviolet light.

A satisfactory design for a glass electrode sterilizable by irradiation is shown in Fig. 2.* A smooth glass seal that must be free from pinholes opening to the surface joins the top of the buffer bridge to the lower end of the reduced taper. The entire electrode, including the inner standard taper, the buffer bridge, and the sensitive bulb, may be wiped clean before sterilization. After irradiation it is transferred to the fermentation flask through sterile air, and sealed at the joint with sterile grease as recommended by Neish and Ledingham (22).

All of the media containing microorganisms filtered from 1400 cu. ft. of laboratory air were irradiated with 60,000 μw. min. per cm.², showed no growth when incubated at 30° C. in the dark for seven days. On subsequent transfer to a brightly lighted laboratory (10) they also remained clear indefinitely at room temperature. All of the unirradiated controls showed heavy bacterial and mold growth.

Contaminated calomel electrodes, made from Leeds and Northrup 1199-27A salt-bridge tubes joined to reduced 24/40 tapers by crevice-free glass seals, after irradiation in air for 60 min. at 1000 μw. per cm.², remained sterile in nutrient broth for one week on incubation at 30° C. It was at first supposed that crevices at the liquid junctions of calomel half-cells would harbor microorganisms, and that the opacity of glass would render sterilization of the salt-bridge tubes impossible by ultraviolet light. Tests in which the empty tubes were autoclaved, and then filled with saturated sterile potassium chloride solution revealed subsequent changes in calibration of as much as several millivolts. This may have been caused by dilution of the solution in channels.

* *Obtainable from Leeds and Northrup Co., Philadelphia, Pa. as electrode 1199-32-S.*

of the liquid junction, or its replacement by water. Saturated aqueous potassium chloride is inherently sterile, and the flow is consistently outward through the junction. It seemed possible, therefore, that microorganisms either did not enter the channels, or were rapidly killed there by osmotic dehydration. This may offer an explanation for the consistent success in sterilizing such calomel electrode assemblies by irradiation.

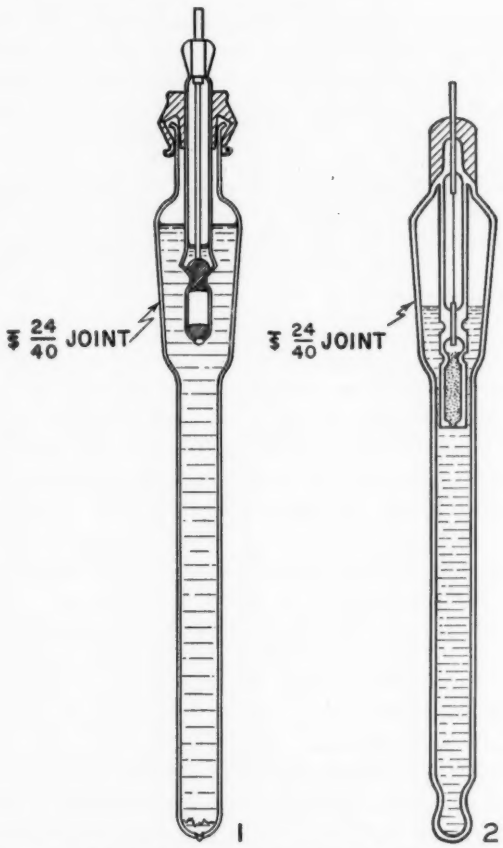


FIG. 1. *Calomel electrode assembly for sterilization by ultraviolet light.*

FIG. 2. *Crevice-free glass electrode sterilizable by irradiation at 2537 Å.*

Calibration tests before and after irradiation of glass-calomel cells exposed to 60,000 $\mu$w. min. per cm.$^2$ with the glass electrode bulbs immersed in water in dishes of Vycor 791 and the calomel electrodes irradiated in air, showed that changes in e.m.f. do not exceed 0.4 mv. corresponding to 0.006 pH units.

## Discussion and Application of Results

The adoption of 60,000 $\mu$w. min. per cm.$^2$ as a standard for the sterilization of electrodes in distilled water is based on the finding that this total energy will inactivate on an agar surface the spores in the largest air-borne clumps of the most resistant organism. The data of Fulton and Coblentz (6), Conklin (3), and Nagy (21) indicate that, of the local air-borne fungi, spores of *A. niger* require the most energy for a 100% kill. Further data are necessary to establish this point beyond dispute. Estimates of the energy at 2537 Å necessary to destroy single spores of *A. niger* on agar agree fairly well. Koller (11) gives about 4950 $\mu$w. min. per cm.$^2$ and Nagy (21) 5600 $\mu$w. min. per cm.$^2$, whereas Conklin (3) reports 0.6% survival after exposure to 8100 $\mu$w. min. per cm.$^2$ The results from Table II show that the survival ratio of single spores in water is $<1 \times 10^{-4}$ after exposure to 7500 $\mu$w. min. per cm.$^2$ Zahl, Koller, and Haskins (30) were unable to detect any significant differences in the energies necessary to kill *A. niger* spores at different relative humidities in air. It is commonly assumed that equivalent total energies are necessary for inactivation, either in water or on an agar surface (8). The energy necessary to inactivate spore clumps on an agar surface (Table III) may, therefore, be assumed to be sufficient to inhibit their development in aqueous suspension.

Evaluation of some of the significant factors shows that selection of a standard such as 60,000 $\mu$w. min. per cm.$^2$ on the basis of tests on a single strain is arbitrary. Moreover, the size and character of the air-borne spore clumps of *A. niger* depend on a series of factors. Among these may be mentioned the substrate on which the organism is grown, the age of the spores, the strain, and the velocity of the air. Observations on cultures of *A. niger* Strain 215 grown on bran and subsequently dried showed that particles of the substrate, with spores attached, became air-borne. The increased energy necessary to inactivate such protected spores was not determined. Unpublished data show that changes in the spore with age, e.g. increases in the secondary thickening and pigmentation of the wall, increase the energy necessary to inhibit its development. Interstrain differences in the structure of the wall, especially the presence of spines and mucilaginous substances, likewise cause variations in the tendency of spores to adhere in clumps. The dependence of rate of settling of air-borne spores on wind velocity, and the distribution of spores by air currents, have been reviewed at length by Wolf and Wolf (29). From this review it seems probable that fairly large particles may be carried by the air currents in air-conditioned buildings. All of these factors make it clearly impossible to assign any absolute value to the total energy at 2537 Å necessary to ensure sterilization of a glass surface exposed to air.

In the light of the previous discussion the use of 60,000 $\mu$w. min. per cm.$^2$ at 2537 Å to sterilize crevice-free electrodes is justified only as part of a strictly defined procedure. Beside the statistical improbability of frequent contamination by the largest air-borne spore clumps of *A. niger* must be set the possibility of bacterial films or particles and also of molds remaining on

electrodes from one fermentation to another. Even if electrodes are carefully cleaned and sterilized after use it has been observed that storage in unsterilized distilled water provides conditions in which microorganisms may grow. If the storage period is prolonged, bacteria and molds may form colonies on the glass bridges. Tests have shown that both bacteria and fungi on these contaminated electrodes will survive exposure for five hours in the sterilizer, i.e. irradiation with a total energy >200,000 $\mu$w. min. per cm.$^2$ Bacteria alone protected in small visible particles show survival and growth after exposure to 78,000 $\mu$w. min. per cm.$^2$

The following procedure, designed to eliminate these hazards, has now been tested in pH controlled fermentation studies for over a year and without exception no failure to maintain cultural purity has occurred. At the end of a fermentation period both of the specially designed crevice-free glass and calomel electrodes are wiped with wet cotton wool and rinsed until the adherent films and visible particles have been removed. Exposure to 60,000 $\mu$w. min. per cm.$^2$ is followed by storage in sterile distilled water in 500 ml. flasks provided with 24/40 standard taper joints. During irradiation the energy may be more uniformly applied if each electrode is turned through 90° halfway through the exposure period (22). Sterile air for the transfer to the flasks is provided by enclosing both the flasks and the sterilizer in a massively irradiated cabinet. On removal from the storage flasks the electrode bulbs are immersed in a buffer of the desired pH to obtain the balanced potentiometer setting on the pH monitor (1). They are then again cleaned, rinsed in *sterile* water, and sterilized by exposure to 60,000 $\mu$w. min. cm.$^2$ The final transfer to the fermentation flasks is made through sterile air.

The variations in potential that accompany superficial drying of Corning 015 type glasses are well known (20) and Dole (4) has called attention to the damage that results when glass electrode bulbs are dried in air at an elevated temperature. Therefore, when bulbs of Corning 015 are in use, it is an advantage to immerse the glass electrode bulbs in water during the irradiation period. However, Perley (24) has recently reported that the newer lithia–silica glasses are much less susceptible to drying influences than are the Corning 015 types. Apart from the drying effect, irradiation in water would appear to favor dispersal, and exposure of microorganisms to the radiation. If the lower ends of the glass electrodes are immersed in distilled water contained in quartz dishes approximately 90% of the energy will reach the submerged bulbs. The exact value will depend on the grade of quartz and the manner in which it has been worked. If Vycor 791 is substituted for quartz the energy transmitted will be near 75% (23). The transmission of the dishes in actual use must be measured. The above estimates allow for surface reflection and solarization (23), and for absorption by the water (16).

Once the total energy necessary to ensure inactivation of the most resistant air-borne organism has been established, the duration of a lethal exposure may be estimated for a given sterilizer. In the sterilizer described (22) the mean, minimum, and maximum intensities at the position of the bulbs are

1340, 680, and 2400 μw. per cm.²; at the four vertical cube faces (see experimental section) 900, 1580, 1050, and 1650 μw. per cm.² respectively. The minimum and average intensities are, therefore, 680 and 1340 μw. per cm.² respectively. These intensities vary significantly with the age of the lamps so that it is necessary to check their energy emission at intervals. An exposure time based on the minimum intensity reaching any part of an electrode surface to be sterilized in water of $\frac{60000}{680} \times \frac{4}{3}$ min., or approximately two hours, is indicated. Simple changes in the design of the sterilizer may allow this period to be cut to 30 min.

Electrode sterilization is one of the critical steps in the application of precision pH control to fermentation media. This investigation has shown that both glass and calomel electrodes may be completely sterilized by irradiation without significantly altering their previous calibration. To achieve this result an electrode design is necessary in which both the glass and calomel half-cells present crevice-free glass surfaces below the reduced tapers used to seal the electrodes to the fermentation flasks. The crevices at the liquid junction of certain calomel salt-bridge tubes form an exception to this rule. They are apparently kept sterile by the outward flow of saturated aqueous potassium chloride.

## Acknowledgments

The authors wish to express their indebtedness to Dr. G. W. Harmsen for invaluable help and advice and to Dr. J. W. Groves and Mr. W. I. Illman for their kindness in identifying the air-borne fungi. They also take pleasure in acknowledging the technical assistance of Mr. S. G. Harbottle and Mr. J. L. Barnwell.

## References

1. Breeze, J. E. A precision electronic pH control. Natl. Research Council Can. Rept. No. ERB-216. 1948.
2. Breeze, J. E. A precision electronic pH control. Proc. Natl. Electronics Conf. 4 : 451-460. 1948.
3. Conklin, D. B. Ultraviolet radiation of spores of certain molds collected from bread. Proc. Iowa Acad. Sci. 51 : 185-189. 1944.
4. Dole, M. The glass electrode. John Wiley & Sons Inc., New York. 1941.
5. Duggar, B. M. and Hollaender, A. Irradiation of plant viruses and of microorganisms with monochromatic light. J. Bact. 27 : 219-239. 1934.
6. Fulton, H. R. and Coblentz, W. W. The fungicidal action of ultraviolet radiation. J. Agr. Research, 38 : 159-168. 1929.
7. Haugaard, G. The action of water on glass. Glastech. Ber. 17 : 104-106. 1939.
8. Hollaender, A. Abiotic and sublethal effects of ultraviolet radiation on microorganisms. Aerobiology. Pub. Am. Assoc. Advancement Sci. 17 : 156-165. 1942.
9. Hollaender, A. Effects of ultraviolet radiation. Ann. Rev. Physiol. 8 : 1-16. 1946.
10. Kelner, A. Photoreactivation of ultraviolet-irradiated *Escherichia coli* with special references to the dose-reduction principle and to ultraviolet-induced mutation. J. Bact. 58 : 511-522. 1949.
11. Koller, L. R. Bactericidal effects of ultraviolet radiation produced by low pressure mercury vapor lamps. J. Applied Phys. 10 : 624-630. 1939.
12. Kratz, L. The susceptibility of electrode glasses to chemical attack. Glastech. Ber. 20: 305-315. 1942.

13. LEA, D. E. and HAINES, R. B. The bactericidal action of ultraviolet light. J. Hyg. 40 : 162-171. 1940.

14. LONGSWORTH, L. G. and MACINNES, D. A. Bacterial growth with automatic pH control. (A) An apparatus. (B) Some tests on the acid production of *Lactobacillus acidophilus*. J. Bact. 29 : 595-607. 1935.

15. LONGSWORTH, L. G. and MACINNES, D. A. Bacterial growth at constant pH. Quantitative studies on the physiology of *Lactobacillus acidophilus*. J. Bact. 31 : 287-300. 1936.

16. LUCKIESH, M. and HOLLADAY, L. L. Disinfecting water by means of germicidal lamps. Gen. Elec. Rev. 47 : 45-50. 1944.

17. LUCKIESH, M., TAYLOR, A. H., and HOLLADAY, L. L. Sampling devices for air-borne bacteria. J. Bact. 52 : 55-65. 1946.

18. LUCKIESH, M. and KNOWLES, T. Resistivity of *Escherichia coli* to ultraviolet energy (λ2537) as affected by irradiation of preceding cultures. J. Bact. 55 : 369-372. 1948.

19. LUYET, B. J. The effects of ultraviolet, X- and cathode rays on the spores of the *Mucoraceae*. Radiology, 18 : 1019-1022. 1932.

20. MACINNES, D. A. and BELCHER, D. Further studies on the glass electrode. J. Am. Chem. Soc. 53 : 3315-3331. 1931.

21. NAGY, R. Control of fungi in bakery plants. Baker's Digest, 22(3) : 25-26 and 29. 1948.

22. NEISH, A. C. and LEDINGHAM, G. A. Production and properties of 2,3-butanediol. XXXII. 2,3-Butanediol fermentations at poised hydrogen ion concentrations. Can. J. Research, B, 27 : 694-704. 1949.

23. NORDBERG, M. E. Ultraviolet-transmitting glasses for mercury vapor lamps. J. Am. Ceram. Soc. 30 : 174-179. 1947.

24. PERLEY, G. A. pH response of glass electrodes. Anal. Chem. 21 : 559-562. 1949.

25. RAHN, O. Physical methods of sterilization of microorganisms. Bact. Revs. 9 : 1-47. 1945.

26. RENTSCHLER, H. C. and NAGY, R. Bactericidal action of ultraviolet radiation on air-borne organisms. J. Bact. 44 : 85-94. 1942.

27. WATSON, R. W., TAMBOLINE, F., and HARMSEN, G. W. Production and properties of 2,3-butanediol. XXXIII. Automatic pH control in the dissimilation of sucrose by *Bacillus polymyxa*. Can. J. Research, F, 27 : 457-469. 1949.

28. WELLS, W. F. and FAIR, G. M. Viability of *B. coli* exposed to ultraviolet radiation in air. Science, 82 : 280-281. 1935.

29. WOLF, F. A. and WOLF, F. T. The fungi. Vol. 2. John Wiley & Sons, Inc., New York. 1947.

30. ZAHL, P. A., KOLLER, L. R., and HASKINS, C. P. The effects of ultraviolet radiation on spores of the fungus *Aspergillus niger*. J. Gen. Physiol. 22 : 689-698. 1939.

# INFLUENCE OF THE AMINO ACID – DEXTROSE REACTION ON GROWTH OF *RHIZOBIUM*[1]

By Dyson Rose and Ruth Peterson


## Abstract

Growth of *Rhizobium*, as measured by turbidity of cultures, was not inhibited appreciably by the brown, fluorescent products formed by the reaction of various amino acids with dextrose. Autoclaving the medium with dextrose actually improved growth of the organism in some trials by partially destroying an amino acid that was inhibitory if present in excess.


## Introduction

Previous work has indicated that the brown, fluorescent materials formed by the reaction of amino acids with reducing sugars were not inhibitory to various species of lactic acid bacteria (7) or to *Bacillus polymyxa* (5). It was therefore of interest to investigate the effects of these materials on a less adaptive organism such as *Rhizobium*.*

## Materials and Methods

A pure strain of *Rhizobium* isolated from clover (supplied by Dr. H. Katznelson, Central Experimental Farm, Ottawa) was used for these studies. Cultures were maintained on agar slants of the composition shown in Table I, Column 1, and transferred to liquid media of the same composition 48 hr. before inoculation of test media. The two test media, designated as "yeast extract" and "casein hydrolyzate", had the compositions shown in Columns 2 and 3 of Table I. Five milliliters of medium was used per tube, and 5 ml. of either 2% dextrose solution or of a solution of dextrose and amino acid – dextrose reaction products was added, either before or after sterilization. Sterilization was accomplished by autoclaving for 13 min. at 121° C., or by filtering through a Type ST, size L6 Seitz pad. One drop of 48-hr. inoculum culture was used to inoculate the 10 ml. of medium thus prepared. All cultures were incubated at 30° C.

Culture tubes were selected to agree within 0.5 galvanometer units when filled with water and examined in the Evelyn colorimeter with a 6600 Å filter, and growth was estimated by determining the transmittance of the culture relative to that of an uninoculated control tube that had received identical treatment. The results are expressed as turbidity, i.e. 100 minus transmittance.

[1] *Manuscript received December 28, 1949.*

*Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as Paper No. 240 of the Canadian Committee on Food Preservation and as N.R.C. No. 2096.*

* *Dr. G. W. Harmsen, of these laboratories, suggested this organism as he had noted that oversterilization of the medium affected it markedly.*

TABLE I

COMPOSITION OF CULTURE MEDIA

| Constituent | Amount used | | |
|---|---|---|---|
| | Stock culture* | Yeast extract** | Casein hydrolyzate** |
| Dipotassium phosphate | 0.5 gm. | 0.5 gm. | 0.5 gm. |
| Magnesium sulphate | 0.2 gm. | 0.2 gm. | 0.2 gm. |
| Sodium chloride | 0.1 gm. | 0.1 gm. | 0.1 gm. |
| Calcium carbonate | 3.0 gm. | 3.0 gm. | 3.0 gm. |
| Yeast extract | 3.0 gm. | 3.0 gm. | — |
| Mannitol | 10.0 gm. | — | — |
| Casein hydrolyzate*** | — | — | 50 ml. |
| Cystine hydrochloride | — | — | 100 mgm. |
| Tryptophan | — | — | 100 mgm. |
| Vitamin solution (Ref. 2) | — | — | 10 ml. |
| Water to make | 1000 ml. | 500 ml. | 500 ml. |

* *Plus 15 gm. agar for slants.*

** *All constituents were dissolved in approx. 200 ml. of glass distilled water, sufficient N sodium hydroxide added to make the mixture alkaline to bromthymol blue, and the volume made to 500 ml. This solution was boiled for a few seconds, cooled overnight, filtered through Whatman No. 2 paper, and readjusted to the desired pH (glass electrode) with acetic acid. This procedure was necessary to avoid the formation of a precipitate during autoclaving.*

*** *General Biochemicals Ltd., Smaco.*

The amino acids used are listed in Tables II and III. Amino acid – dextrose reaction products were prepared by subjecting a solution of dextrose and the desired amino acid to a temperature of 140° F. for several days. The relative concentration of the brown, fluorescent materials in the media was estimated by transmittance and fluorescence readings made on control tubes. Transmittance was determined at a wave length of 4200 Å in the Evelyn instrument, the medium for which the dextrose had been autoclaved separately being used as the control (100% transmittance). Fluorescence was determined in the Coleman, Model 12 photofluorometer with B-1 and PC-1 filters and a quinine sulphate standard.

## Results

### *Effect of pH*

The effect of pH of the culture media on growth of this organism was determined in media autoclaved with dextrose present and in media for which the dextrose had been autoclaved separately. pH was determined by means of a glass electrode in uninoculated media at the conclusion of a 96-hr. incubation period.

Results obtained with yeast extract media at the end of the 48- and 96-hr. growth periods are presented in Fig. 1. The organism appeared to have a relatively narrow pH optimum at pH 6.8 to 7.3, but growth was curtailed less

in acid media that had been autoclaved with dextrose than in acid media for which the dextrose has been autoclaved separately. Similar results were obtained with casein hydrolyzate media.

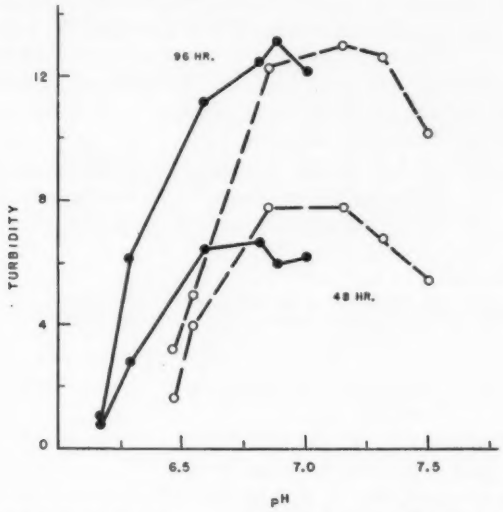


FIG. 1. *Effect of pH of medium on growth of Rhizobium. Solid line—medium autoclaved with dextrose present. Broken line—medium and dextrose autoclaved separately.*

*Effect of Sterilization Procedures*

Under the conditions used in these experiments, autoclaving the media with dextrose resulted in a pH drop of about 0.35 units (range 0.27 to 0.48). Autoclaving casein hydrolyzate media without dextrose increased the pH unless they were made alkaline initially. Autoclaving yeast extract media without dextrose did not alter the pH significantly.

If the media were adjusted to pH 6.8, poorer growth was usually obtained after autoclaving with dextrose present than after autoclaving the dextrose separately. This effect, however, seemed to be entirely due to changes in pH during autoclaving and when allowance for the change in pH was made (by adjusting the portion of medium to be autoclaved with dextrose to pH 7.2) the presence or absence of dextrose during autoclaving had no detectable effect. Similarly, at equal pH after sterilization, the rate of growth in media sterilized by filtration did not differ significantly from that in autoclaved media.

*Effect of Added Amino Acid – Dextrose Reaction Products*

Solutions of amino acid – dextrose reaction products were prepared from casein hydrolyzate, and from several individual amino acids, and added to

the culture media. In addition, tubes containing additional amounts of dextrose, of casein hydrolyzate, and of the individual amino acids were prepared as controls.

The addition of dextrose, up to double the usual concentration, or of many of the individual amino acids, had no significant effect on the rate of growth of the organism. DL-serine, DL-isoleucine, and cystine, on the other hand, appeared to have slight inhibitory effects; and DL-alanine, DL-phenylalanine, DL-threonine, DL-valine, DL-methionine, glycine, and casein hydrolyzate markedly inhibited growth of the organism.

The data presented in Table II show that addition of the brown, fluorescent products formed from the noninhibitory amino acids had no effect on growth of the organism. To distinguish between the effects of the inhibitory amino

TABLE II

GROWTH OF *Rhizobium* IN THE PRESENCE OF BROWN FLUORESCENT PRODUCTS FORMED BY THE REACTION OF VARIOUS AMINO ACIDS WITH DEXTROSE

| Amino acid added | Relative fluorescence | Transmission, 4200 Å | Turbidity | |
|---|---|---|---|---|
| | | | 48 hr. | 96 hr. |
| None | 22.5 | 100.0 | 5.0 | 10.2 |
| Cysteine | 40.5 | 48.5 | 6.1 | 11.2 |
| Histidine | 320.0 | 32.0 | 5.3 | 10.8 |
| Proline | 34.0 | 56.0 | 4.8 | 9.5 |
| None | 23.0 | 100.0 | 4.8 | 9.7 |
| Arginine | 62.0 | 66.0 | 4.2 | 10.8 |
| Aspartic acid | 53.0 | 50.0 | 5.3 | 11.3 |
| Leucine | 37.0 | 65.0 | 5.0 | 12.7 |
| None | 20.5 | 100.0 | 5.7 | 12.0 |
| Tyrosine | 28.5 | 58.0 | 6.0 | 13.0 |
| Tryptophan | 64.0 | 59.5 | 6.2 | 13.5 |
| Lysine | 33.5 | 71.0 | 4.7 | 10.5 |
| Glutamic acid | 47.0 | 55.5 | 5.8 | 12.0 |

acids, and the products of their reaction with dextrose, growth of the organism was followed in tubes "A" containing a known increment of amino acid and in tubes "B" containing the reaction products. The amount of reacted amino acid – dextrose mixture added to tubes "B" was such that these tubes would have contained the same increment of amino acid as tubes "A" if none had been destroyed in the formation of the reaction products.

Table III shows the turbidity of these cultures after incubation for 48 and 96 hr., and also the relative fluorescence and optical density of the uninoculated controls. Considerable inhibition is apparent in tubes "B", containing the amino acid – dextrose reaction products, but in almost every instance the inhibition is less than that caused by the amino acids alone (Tubes "A"). It must be concluded, therefore, that the brown fluorescent materials are noninhibitory or, at least, are less inhibitory than the amino acids themselves.

TABLE III

EFFECT OF AMINO ACIDS AND THE PRODUCTS OF THEIR REACTION WITH DEXTROSE ON GROWTH OF *Rhizobium*

| Amino acid added | Extra amount present* | Fluorescence | | Relative transmission | | Turbidity, 96 hr. | |
|---|---|---|---|---|---|---|---|
| | | Tubes A** | Tubes B*** | Tubes A | Tubes B | Tubes A | Tubes B |
| None | — | 20.0 | — | 100.0 | — | 8.2 | — |
| Casein hydrolyzate | 0.5 ml. | 29.0 | 145.0 | 90.5 | 31.0 | 0.0 | 5.5 |
| DL-alanine | 2.5 mgm. | 22.5 | 62.0 | 99.0 | 42.5 | 0.2 | 3.0 |
| DL-phenylalanine | 2.5 mgm. | 22.0 | 61.5 | 99.5 | 53.5 | 0.3 | 3.2 |
| DL-threonine | 2.5 mgm. | 22.0 | 76.0 | 100.0 | 64.5 | 0.7 | 6.5 |
| DL-valine | 2.5 mgm. | 22.0 | 62.5 | 99.5 | 46.0 | 2.3 | 2.0 |
| Glycine | 0.57 mgm. | 19.5 | 31.5 | 100.0 | 80.5 | 7.2 | 9.2 |
| Glycine | 1.14 mgm. | 19.0 | 44.0 | 100.0 | 67.0 | 3.2 | 7.2 |

* *Amount added, uncorrected for destruction by reaction with dextrose. The amounts of these amino acids present in the basal medium were not determined.*

** *Containing added amino acids.*

*** *Containing amino acid – dextrose reaction products.*

As a further test of the toxicity of the reaction products, the residual glycine in a reacted glycine – dextrose mixture was estimated by a Van Slyke amino nitrogen determination and graded amounts were added to the media. Control tubes, to which pure glycine was added, were also prepared. The initial glycine content of the media was not determined. The data obtained are presented in Fig. 2 (the abscissa scale refers to added glycine only) and indicate that the brown materials exerted a slight inhibitory effect in excess of

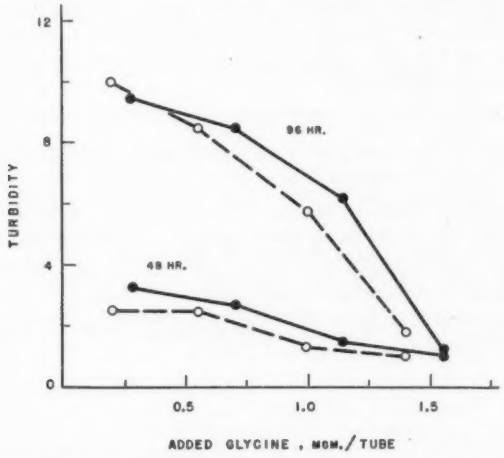


FIG. 2. *Effect of glycine and a reacted glycine – dextrose mixture on growth of Rhizobium. Solid line—glycine alone. Broken line—reacted glycine – dextrose mixture.*

that exerted by glycine alone. The maximum difference, however, is only 1.6 turbidity units after 96 hr. of growth and it is doubtful whether this exceeds the normal variability of results.

## Discussion

Since preparation of our earlier report (7) a paper by Boyd *et al.* (1) has appeared in which it is claimed that *Clostridium perfringens* (*welchii*) is inhibited by substances formed by autoclaving the medium in the presence of glucose, at pH 7.2, but no proof that the inhibition was not due to loss of an essential nutrient is presented. Langford and Lacy (3), on the other hand, have presented additional evidence to show that destruction of nutrients during autoclaving may affect growth of various organisms. We conclude, therefore, that the brown materials formed during autoclaving of media containing organic nitrogen are not toxic to microorganisms and that the apparent inhibitory effects observed by some workers (1, 9) have resulted from destruction of a nutrient that was present in limiting quantity and that reacted with dextrose under the conditions of autoclaving. Evidence has been published showing that cystine (6), cysteine (6), lysine (4), methionine (4), tryptophan (4, 7), and probably some vitamins (3, 4) may be destroyed to such an extent that growth is impaired. Other nutrients can probably behave similarily. This conclusion cannot be extended to media such as those used for the culture of *Cytophaga*, which contain no organic nitrogen and in which an inhibition due to caramelized dextrose has been reported (8).

## References

1. Boyd, M. J., Logan, M. A., and Tytell, A. A. The growth requirements of *Clostridium perfringens* (*welchii*) BP6K. J. Biol. Chem. 174 : 1013-1025. 1948.
2. Henderson, L. M. and Snell, E. E. A uniform medium for determination of amino acids with various microorganisms. J. Biol. Chem. 172 : 15-31. 1948.
3. Langford, C. E. and Lacy, H. Destruction of nutrient components in culture media autoclaved with glucose. Soc. of Am. Bacteriologists, 49th General Meeting, May, 1949. Abstracts, p. 34.
4. Patton, A. R. and Hill, E. G. Inactivation of nutrients by heating with dextrose. Science, 107 : 68-69. 1948.
5. Peterson, R., Rose, D., and Loeb, L. Effect of the amino acid – dextrose reaction on growth of *Bacillus polymyxa*. Can. J. Research, C, 27 : 269-273. 1949.
6. Rabinowitz, J. C. and Snell, E. E. The vitamin B6 group XI: an improved method for assay of vitamin B6 with *S. faecalis*. J. Biol. Chem. 169 : 631-642. 1947.
7. Rose, D. and Peterson, R. Influence of the amino acid – dextrose reaction on growth of lactic acid bacteria. Can. J. Research, B, 27 : 428-436. 1949.
8. Sijpesteijn, A. K. and Fahraeus, G. Adaptation of *Sporocytophaga myxococcoides* to sugars. J. Gen. Microbiol. 3 : 224-235. 1949.
9. Toennies, G. and Gallant, D. L. Bacterimetric studies. I. Factors affecting the precision of bacterial growth responses and their measurement. J. Biol. Chem. 174 : 451-463. 1948.

# THE INTERMEDIATE METABOLISM OF *PSEUDOMONAS AERUGINOSA*

## IV. THE ABSENCE OF AN EMBDEN–MEYERHOF SYSTEM AS EVIDENCED BY PHOSPHORUS DISTRIBUTION[1]

By Jack J. R. Campbell[2] and Flora C. Norris[3]


### Abstract

The acid soluble phosphorus fraction of cells of *Pseudomonas aeruginosa* harvested from a glucose medium was found to consist mainly of difficultly hydrolyzable compounds. None of the fractions contained reducing compounds even after 12 hr. hydrolysis with normal hydrochloric acid nor could fructose be detected. It could therefore be concluded that glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, and hexose diphosphate were absent. Analysis of a glucose solution that had been metabolized by cells of this organism under conditions of fluoride inhibition confirmed the analysis of the cellular material. The lack of normal intermediates of the Embden–Meyerhof scheme is discussed in relation to the gluconic acid, 2-ketogluconic acid pathway of glucose oxidation.


Although little is known of the aerobic breakdown of carbohydrates by microorganisms it is generally assumed that under these conditions carbohydrate dissimilation follows the Embden–Meyerhof pathway to the pyruvate stage and further breakdown proceeds either by way of a cyclic system such as the Kreb's cycle or by direct degradation. In either case the end products are carbon dioxide and water. The assumption that the initial breakdown proceeds by way of an anaerobic mechanism in the case of facultative organisms is in accord with our present knowledge. Nevertheless in various studies on aerobic cellular metabolism sufficient data have appeared to question the assumption that the normal anaerobic mechanism is the only means of initiating the breakdown of the carbohydrate molecule (1, 3, 5, 6, 12, 13, 14, 16). The fragmentary nature of these data reveals little of the importance of the systems studied to the metabolism of the cell and furnishes no coherent picture of the oxidative pathway of carbohydrate metabolism.

Since it has been demonstrated that unphosphorylated gluconic and 2-ketogluconic acids are intermediates in the oxidation of glucose by the obligate aerobe *Pseudomonas aeruginosa* (18), it was of interest to determine whether or not glucose-6-phosphate was present as an intermediate and was thus a possible precursor of gluconic acid and whether any of the other intermediates of the Embden–Meyerhof pathway were formed during glucose oxidation. This information would greatly assist in deciding whether or not the pathway of glucose oxidation of this organism is made up of any of the reactions known

[1] *Manuscript received December 5, 1949.*

*Contribution from the Department of Dairying, The University of British Columbia, Vancouver, B.C. This study was carried out under a grant from the National Research Council of Canada, laboratory facilities being found by The University of British Columbia.*

[2] *Associate Professor of Dairying.*

[3] *Graduate Student.*

to occur in the Embden–Meyerhof or other known systems. It would also permit a more accurate assessment of the importance of the gluconic acid, 2-ketogluconic acid pathway in the metabolic scheme of *P. aeruginosa*.

## Methods

To obtain cells for analysis, 3 liters of glucose ammonium phosphate medium (4) dispensed in 100-ml. quantities in Roux flasks were inoculated with a 24 hr. culture of *P. aeruginosa A.T.C. 9027* and incubated at 30° C. for 24 hr. The cells were collected in a Sharples centrifuge and the resulting paste was rapidly dried in a vacuum desiccator. A yield of 1.1 gm. of dried cells was obtained by this procedure. The fractionation procedure employed was a modification of that used by LePage and Umbreit (11). The 1.1 gm. of dried cells were suspended in 7.5 ml. of distilled water, treated for five minutes with 0.75 ml. of 50% potassium hydroxide and then neutralized with 100% trichloroacetic acid. To the neutralized suspension 1.25 ml. of 100% trichloroacetic acid was added and the total volume of the mixture made to 12.5 ml. with water. The extraction was then carried out for 16 hr. at 0° to 5° C. The extract was centrifuged and the supernatant set to one side. Ten ml. of 10% trichloroacetic acid was added to the cell debris and a second extraction at 0° to 5° C. was carried out for 48 hr. The extract was again centrifuged and the cell debris was subjected to three additional extractions with 10% trichloroacetic acid. Further extraction did not result in the removal of any additional phosphate. The extracts were combined and made to a volume of 50 ml.

Analysis of the extract showed that almost half of the phosphorus present occurred as inorganic phosphate. Since inorganic phosphate would interfere with subsequent determinations it was removed as magnesium ammonium phosphate by adjusting the extract to pH 8.2 with ammonium hydroxide and adding magnesium acetate until no further precipitation occurred. The precipitate containing all the inorganic phosphate was removed by filtration.

To 50 ml. of the filtrate, which had been freed of inorganic phosphate, 2.7 ml. of 25% barium acetate was added and the mixture allowed to stand overnight at 0° to 5° C. The barium insoluble fraction was removed by centrifugation, washed with water, and dried in a vacuum desiccator. A slight excess of sulphuric acid was added to the supernatant and the precipitated barium sulphate was removed and washed twice with a small volume of water. The precipitate was discarded and the washings added to the supernatant. A second addition of barium acetate was made to the supernatant and the pH adjusted to 8.2 with sodium hydroxide. After standing at 0° to 5° C. for 30 min., the second barium insoluble precipitate was removed, washed, and subsequently dried in a vacuum desiccator. Since this precipitate contained considerable barium sulphate it was not combined with the first barium insoluble fraction. Five volumes of ice-cold 95% ethyl alcohol were added to the supernatant. After standing overnight at 0° to 5° C. the mixture was centrifuged, the precipitate washed, and then dried in a vacuum desiccator.

The neutral supernatant was reduced to a volume of 15 ml. by vacuum distillation. By this procedure four fractions were obtained: the first barium insoluble fraction; the second barium insoluble fraction; the barium soluble, alcohol insoluble fraction; and finally the barium soluble, alcohol soluble fraction.

Barium was removed from the first barium insoluble fraction, the barium soluble, alcohol insoluble fraction and the barium soluble, alcohol soluble fraction by the addition of a slight excess of sulphuric acid. In each case the barium sulphate was washed three times and washings and supernatant were combined and made to the desired volumes. The first barium insoluble fraction, the second barium insoluble fraction, and the barium soluble, alcohol soluble fractions were each made to a volume of 25 ml. while the barium soluble, alcohol insoluble fraction was made to a volume of 50 ml.

Analyses of the fractions were carried out by the following methods: total and inorganic phosphate were determined by the method of King (10); reducing value by the method of Shaffer and Somogyi (22); fructose by the method of Roe (21); pentose by the method of Meijbaum (15); total nitrogen by a modification of the method of Umbreit, Burris, and Stauffer (23); and phosphoglyceric acid by the method of Rapoport (20). Hydrolysis curves were run in $N/1$ hydrochloric acid at 100° C. in a boiling water bath.

In order to obtain additional information the procedure that Dubois and Potter (7) had used in isolating phosphate esters from bakers' yeast was applied using 16 gm. of cell paste obtained from a 24 hr. culture of *P. aeruginosa* grown on glucose ammonium phosphate medium. Using this procedure, only the barium insoluble phosphate fraction could be isolated. After being dried in a vacuum desiccator the precipitate was treated with a slight excess of sulphuric acid and the precipitated barium sulphate washed three times with water. The supernatant and washings were made to 50 ml. The barium insoluble fraction obtained by this procedure was analyzed for organic phosphorus, reducing value, fructose, pentose, nitrogen, and phosphoglyceric acid. The rate of acid hydrolysis of the phosphate fraction was also determined.

## Experimental

The distribution of the acid soluble organic phosphorus fraction in cells of *P. aeruginosa* was found to differ markedly from the findings reported for *Thiobacillus thioxidans*, an organism that has an Embden–Meyerhof system. For comparative purposes values for *T. thioxidans* as obtained by LePage and Umbreit (11) are included in Table I. In the case of *P. aeruginosa* over 50% of the phosphorus occurred in the barium insoluble fraction while with *T. thioxidans* nearly 70% occurred in the alcohol insoluble fraction.

More detailed information on the nature of each of the fractions was obtained by following the release of inorganic phosphorus during hydrolysis by $N/1$ hydrochloric acid at 100° C. (Fig. 1).

TABLE I

THE DISTRIBUTION OF ACID SOLUBLE PHOSPHORUS IN BACTERIAL CELLS

| Fraction | *P. aeruginosa* | | *Thiobacillus thioxidans** |
|---|---|---|---|
| | Phosphorus in each fraction, mgm. | % of organic phosphorus | % of organic phosphorus |
| Original extract | 10.0 | | |
| 1st Ba insoluble | 2.427 | 24.27 | |
| 2nd Ba insoluble | 3.00 | 30.00 | |
| Total Ba insoluble | 5.427 | 54.27 | 23.7 |
| Ba soluble, alcohol insoluble | 3.783 | 37.83 | 67.6 |
| Ba soluble, alcohol soluble | .518 | 5.18 | 3.4 |
| % recovery | | 97.28 | 94.7 |

**From LePage and Umbreit (11).*

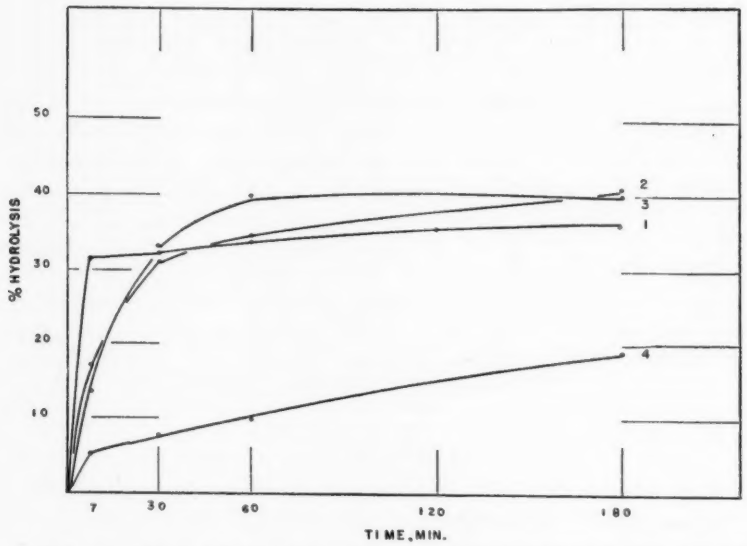


FIG. 1. *Hydrolysis of organic phosphorus compounds in fractions obtained from cells of P. aeruginosa. Curve 1, first barium insoluble fraction; Curve 2, second barium insoluble fraction; Curve 3, barium soluble, alcohol insoluble fraction; and Curve 4, barium soluble, alcohol soluble fraction.*

Continuation of the hydrolysis for a period of 12 hr. resulted in the liberation of little additional phosphorus. Hydrolysis curves for control solutions of glucose-1-phosphate and glucose-6-phosphate were established and the results were found to agree with those reported in the literature. The reducing

values of the various fractions and of the glucose phosphates were determined at the same time that samples were removed for the hydrolysis curves.

From the curves recorded in Fig. 1, it can be seen that approximately 60% of the organic phosphorus in the two barium insoluble and in the alcohol insoluble fraction is resistant to acid hydrolysis. Approximately 80% of the phosphorus in the barium soluble, alcohol soluble fraction is resistant to acid hydrolysis. The hydrolysis curves for the barium insoluble fractions are distinctly different from those for the comparable fraction from *T. thioxidans* (11). No fraction was found to have an initial reducing value, nor was there any increase in the reducing values after hydrolysis with the exception of the first barium insoluble fraction, which was found to give a slight reduction of the copper reagent after three hours hydrolysis. Since this reduction of copper was not correlated with phosphorus release it was considered that the reduction was not related to any of the compounds discussed below. On the basis of reducing values it can be concluded that hexose-diphosphate, glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate are absent from the trichloroacetic acid extract of glucose-grown cells of *P. aeruginosa*. Hexose diphosphate, which is normally present in the barium insoluble fraction, would give a small initial reducing value, which increases with hydrolysis. Glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate are normally found in the alcohol insoluble fraction. Glucose-1-phosphate gives no initial reduction but is completely hydrolyzed to glucose after seven minutes at 100° C. in *N*/1 hydrochloric acid. Thus a reducing reaction would be obtained after seven minutes' hydrolysis if the compound were present. Glucose-6-phosphate is initially reducing and since it is resistant to hydrolysis there is little increase in reduction due to hydrolysis. Fructose-6-phosphate also gives an initial reducing value that increases considerably on hydrolysis.

Confirmation of the absence of hexose diphosphate and fructose-6-phosphate was obtained when no trace of fructose could be detected in any of the fractions. Pentose determinations were carried out on each fraction and values for pentose, total phosphorus, and seven minute phosphorus are recorded in Table II. The value obtained for pentose recovery was 100.3% of that determined in the original extract.

TABLE II

DISTRIBUTION OF TOTAL PHOSPHORUS, SEVEN MINUTE PHOSPHORUS, AND PENTOSE

| Fraction | Volume of fraction, ml. | Pentose, μgm./ml. | Total P, μgm./ml. | Seven minute, μgm./ml. |
|---|---|---|---|---|
| 1st Ba insoluble | 25 | 50 | 95 | 30 |
| 2nd Ba insoluble | 25 | 170 | 120 | 20 |
| Ba soluble, alcohol insoluble | 50 | 120 | 75 | 10 |
| Ba soluble, alcohol soluble | 25 | 22 | 20 | <1 |

Nitrogen values determined for all fractions were considerably higher than could be accounted for as known nitrogen-containing compounds. Analysis of the original trichloroacetic extract using paper chromatography revealed the presence of small amounts of ninhydrin-reacting substances and consequently it was concluded that the presence of amino acids, dipeptides, or other ninhydrin reacting substances was responsible for the high nitrogen values obtained. Thus nitrogen values could not be used in estimating adenosine triphosphate, adenosine diphosphate, coenzyme I, coenzyme II, or adenylic acid. The minor importance of adenosine phosphates and coenzymes in a study such as this did not warrant their determination by more detailed procedures. Estimations based on pentose and hydrolyzable phosphorus values revealed that with the exception of the second barium insoluble fraction, pentose could be accounted for as nitrogenous compounds known to occur normally in the respective fractions. In the second barium insoluble fraction, however, it was found that, in relation to the amount of seven minute phosphorus determined, the pentose value was approximately twice that required for adenosine diphosphate or adenosine triphosphate. It is possible that the excess pentose occurs as a difficultly hydrolyzable pentose phosphate. The hydrolysis curve for this fraction would support this suggestion. Phosphoglyceric acid could not be detected in either of the barium insoluble fractions.

Confirmation of the absence of the initial compounds of the Embden–Meyerhof scheme was obtained when the procedure of Dubois and Potter (7) for the isolation of phosphate esters was carried out with cells of *P. aeruginosa*. If this organism has an Embden–Meyerhof system, then by this procedure it should be possible to isolate glucose-6-phosphate, hexose-diphosphate, and phosphoglyceric acid. The procedure differs from that just discussed in that it makes use of fluoride to cause the accumulation of phosphate esters and the material for analysis is not the bacterial cells but the glucose solution that the culture has been metabolizing. Toluene is used to aid in releasing the phosphorus derivatives from the cells and the extraction period with trichloroacetic acid is 20 min. rather than the complete extraction of the first procedure.

In carrying through this procedure only the barium insoluble fraction was detected. In an organism with an Embden–Meyerhof scheme this fraction would contain a mixture of hexose diphosphate and phosphoglyceric acid. The lack of an alcohol insoluble fraction may be taken as further evidence of the absence of glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate in the pathway of glucose degradation by *P. aeruginosa*. The barium insoluble fraction was analyzed by the methods used for fractions of the cell extract. Phosphorus liberation for the fraction on hydrolysis with $N/1$ hydrochloric acid at 100° C. is shown in Fig. 2. Continued hydrolysis for 12 hr. gave little increase in inorganic phosphorus.

Again it can be seen that a high percentage of the organic phosphorus is resistant to hydrolysis. Reducing values, carried out parallel with phosphorus determinations once more indicated the absence of hexose diphosphate. Negative tests for fructose confirmed the absence of hexose diphosphate from

this fraction. Pentose was determined on the fraction, and all pentose can be accounted for as adenosine phosphates. Again estimations are based on seven minute phosphorus since nitrogen values were excessively high.

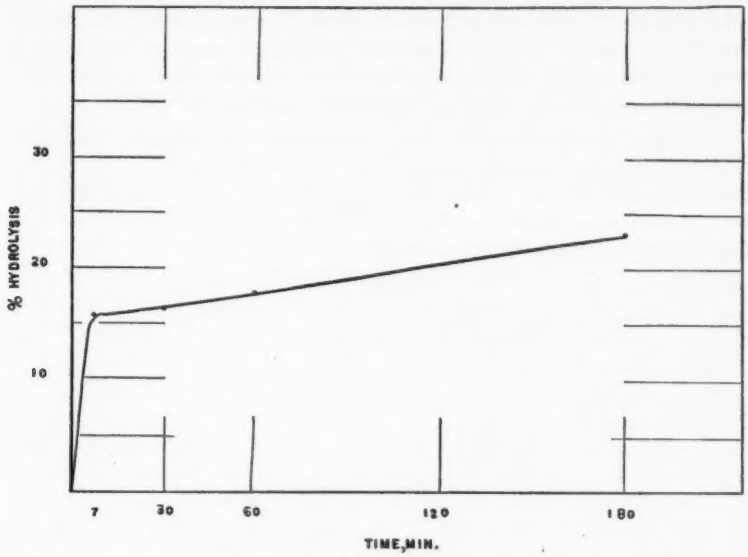


FIG. 2. *Hydrolysis of organic phosphorus compounds in barium insoluble fraction. (Dubois and Potter procedure (7).)*

The fraction obtained by this procedure differed from the barium insoluble fractions obtained from dried cells in one respect. It was found that this fraction contained relatively high amounts of a substance giving a reaction for phosphoglyceric acid.

TABLE III

ANALYSIS OF THE BARIUM INSOLUBLE FRACTION OBTAINED BY THE PROCEDURE OF DUBOIS AND POTTER

| Substance | μgm./ml. |
|---|---|
| Total phosphorus | 75 |
| P liberated after seven minutes at 100° C. with *N*/1 HCl | 12.5 |
| Increase in reducing value after 12 hr. hydrolysis | 0 |
| Pentose | 44 |
| "Phosphoglyceric acid phosphorus" | 33.3 |

## Discussion

Previous work on the metabolism of *P. aeruginosa* has indicated that this organism is unique in a number of respects. It would appear that its storage products differ from those of most other organisms in that they are oxidized

at a constant rate irrespective of the presence or absence of oxidizable substrate and that the endogenous respiration cannot be lowered either by storage at low temperatures or by aeration for eight hours. Also it has been shown that the degree of oxidative assimilation is not affected by dinitrophenol (19). In addition it has been found that under normal physiological conditions free gluconic and 2-ketogluconic acids are formed as intermediate products during the oxidation of glucose and constitute steps in a pathway not previously reported in any type of tissue or bacterial cell (18).

In the present work it has been shown that neither the fermentation liquor nor the cells of this organism contain any of the hexose members of the Embden–Meyerhof scheme. This information confirms and extends previous observations made on the metabolism of this organism and allows a more far reaching appraisal of its pathway of glucose oxidation.

The hydrolysis curves of the phosphorylated intermediates indicate the presence of a group of compounds that are much more resistant to acid hydrolysis than any of the compounds of the Embden–Meyerhof scheme. This information serves to re-emphasize the major role that this new pathway must play in the metabolism of *P. aeruginosa* and indicates how different it is from other pathways. An additional feature of the scheme is that no derivatives of fructose are detectable. Further evidence that this organism does not initiate the dissimilation of glucose by way of the Embden–Meyerhof pathway was obtained when it was found that under anaerobic conditions resting cell suspensions failed to remove any glucose or fructose or to take up phosphate (17).

The absence of any of the hexose monophosphates extends the observation (20) that the storage products of this organism differ from those normally found in microorganisms. That is if "glycogen" is the storage product one would expect glucose-1-phosphate and glucose-6-phosphate to be present either in the cells or in the fermentation liquor; yet neither of these compounds could be detected.

The possibility that phosphoglyceric acid was formed during the oxidation of glucose was revealed by the positive Rapoport test on the barium insoluble fraction obtained from the fermentation liquor in the Dubois and Potter procedure. However this test is not sufficiently specific to allow any conclusions to be drawn. Moreover the positive test was obtained only in the presence of sodium fluoride. The status of phosphoglyceric acid must await further work making use of the optical rotation of the compound.

The evidence that *P. aeruginosa* oxidizes glucose without forming any of the hexose members of the Embden–Meyerhof system confirms the conclusion that the pathway through gluconic and 2-ketogluconic acids is of major importance to this organism and is probably its only means of oxidizing glucose. These data agree with Barron's statement that *P. aeruginosa* oxidizes glucose without first phosphorylating it (2). However phosphorylated intermediates are certainly formed, at least during the later stages of oxidation.

The oxidation of glucose without concurrent phosphorylation casts some doubts on the efficiency of the system in yielding energy. However it is conceivable that the oxidation of glucose to gluconic and 2-ketogluconic acids is similar to the oxidation of the reduced form of diphosphopyridine nucleotide. Friedkin and Lehninger (8) have shown that hydrogen transport between D.P.N.$H_2$ and oxygen can be coupled with phosphorylation of a second system. By a system such as this, glucose could be oxidized to gluconic acid while an entirely different compound was phosphorylated during the oxidation. A similar reaction resulting perhaps in the phosphorylation of amino acids or of part of the energy source could account for the greatly increased synthesis that is part of the Pasteur effect and the lower than theoretical oxygen uptake that accompanies oxidative assimilation.

The pathway of degradation of glucose by *P. aeruginosa* appears to be somewhat similar to that proposed by Dickens for yeast (5, 6). Dickens stated that yeast may oxidize glucose by way of phosphogluconic and phospho-2-ketogluconic acids and that the breakdown was a stepwise procedure through 5-carbon, 4-carbon, and eventually 3-carbon compounds. Our experiments have shown that *P. aeruginosa* oxidizes glucose by way of gluconic and 2-ketogluconic acids and that a pentose phosphate may be formed. There is also evidence that only one mole of acetate is formed from every mole of glucose oxidized (4). These data may be interpreted to mean that here too there is a stepwise breakdown of glucose without the conventional 3–3 split. Although the scheme of oxidation proposed by Dickens does involve phosphorylated intermediates the phosphate is on the sixth carbon while the oxidation occurs at the first with the result that the phosphorylation seems to take no part in energy transfer. The absence in the present work of phosphorylated intermediates with reducing activity or of compounds that, on acid hydrolysis, yield a reducing compound is additional evidence that *P. aeruginosa* does not form phospho-2-ketogluconic acid as an intermediate in glucose oxidation. The possibility that phosphogluconic and phospho-2-ketogluconic acids are formed and are rapidly broken down by an active phosphorylase is ruled out by the absence of glucose-6-phosphate, which is a precursor to these compounds in every known system.

It seems most probable that the dissimilation of glucose by *P. aeruginosa* is initiated either by the glucose dehydrogenase described by Harrison (9) for mammalian liver or by the glucose oxidase described by Muller (16) for *A. niger*. In either case gluconic acid is formed. This would mean that 2-ketogluconic acid is formed by a simple dehydrogenation of gluconic instead of by way of phosphogluconic as is usually accepted. The elucidation of the steps beyond this point must await a more detailed analysis of the phosphorylated intermediates.

## Acknowledgment

The authors wish to express their appreciation to Dean B. A. Eagles for his helpful suggestions and criticism of the manuscript.

## References


1. BARKER, S. B., SHORR, E., and MALAM, M. Studies on the Pasteur reaction: effect of iodoacetic acid on the carbohydrate metabolism of isolated mammalian tissues. J. Biol. Chem. 129 : 33-50. 1939.
2. BARRON, E. S. G. Mechanisms for the complete oxidation of carbohydrates by aerobic bacteria. Symposium on respiratory enzymes. University of Wisconsin Press. 1941.
3. BARRON, E. S. G. and FRIEDEMANN, T. E. Studies on biological oxidations. XIV. Oxidations by microorganisms which do not ferment glucose. J. Biol. Chem. 137 : 593-610. 1941.
4. CAMPBELL, J. J. R., NORRIS, F. C., and NORRIS, M. E. The intermediate metabolism of *Pseudomonas aeruginosa*. II. Limitations of simultaneous adaptation as applied to the identification of acetic acid, an intermediate in glucose oxidation. Can. J. Research, C, 27 : 165-171. 1949.
5. DICKENS, F. Mechanism of carbohydrate oxidation. Nature, 138 : 1057. 1936.
6. DICKENS, F. Oxidation of phosphohexonate and pentose phosphoric acids by yeast enzymes. I. Oxidation of phosphohexonate. Biochem. J. 32 : 1626-1636. 1938.
7. DUBOIS, K. P. and POTTER, V. R. Preparation of hexose diphosphate, hexose monophosphate and phosphoglyceric acid. J. Biol. Chem. 147 : 41-46. 1943.
8. FRIEDKIN, M. and LEHNINGER, A. L. Phosphorylation coupled to electron transport between dihydrogen diphosphopyridine nucleotide and oxygen. J. Biol. Chem. 174 : 757-758. 1948.
9. HARRISON, D. C. Glucose dehydrogenase: a new oxidizing enzyme from animal tissues. Biochem. J. 25 : 1016-1027. 1931.
10. KING, E. J. The colorimetric determination of phosphorus. Biochem. J. 26 : 292-297. 1932.
11. LEPAGE, G. A. and Umbreit, W. W. Phosphorylated carbohydrate esters in autotrophic bacteria. J. Biol. Chem. 147 : 263-271. 1943.
12. LIPMANN, F. Fermentation of phosphogluconic acid. Nature, 138 : 588. 1936.
13. LOCKWOOD, L. B. and STODOLA, F. H. Preliminary studies on the production of $\alpha$-ketoglutaric acid by *Ps. fluorescens*. J. Biol. Chem. 164 : 81-83. 1946.
14. LOCKWOOD, L. B., TABENKIN, B., and WARD, G. E. The production of gluconic acid and 2-ketogluconic acid from glucose by species of *Pseudomonas* and *Phytomonas*. J. Bact. 42 : 51-61. 1941.
15. MEIJBAUM, W. Estimation of small amounts of pentose especially in derivatives of adenylic acid. Z. physiol. Chem. 258 : 117-120. 1939.
16. MULLER, D. Studien über ein neues Enzym Glucose-oxydase. Biochem. Z. 199 : 136-170. 1928.
17. NEY, P. W. Thesis. The University of British Columbia. 1948.
18. NORRIS, F. C. and CAMPBELL, J. J. R. The intermediate metabolism of *Pseudomonas aeruginosa*. III. The application of paper chromatography to the identification of gluconic and 2-ketogluconic acids, intermediates in glucose oxidation. Can. J. Research, C, 27 : 253-261. 1949.
19. NORRIS, F. C., CAMPBELL, J. J. R., and NEY, P. W. The intermediate metabolism of *Pseudomonas aeruginosa*. I. The status of the endogenous respiration. Can. J. Research, C, 27 : 157-164. 1949.
20. RAPOPORT, S. A modified method for the determination of phosphoglyceric acid by the step-photometer. Biochem. Z. 291 : 429-432. 1937.
21. ROE, J. H. A colorimetric method for the determination of fructose in blood and urine. J. Biol. Chem. 107 : 15-22. 1934.
22. SHAFFER, P. A. and SOMOGYI, M. Copper iodometric reagents for sugar determination. J. Biol. Chem. 100 : 695-713. 1933.
23. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. Manometric techniques and related methods for the study of tissue metabolism. Burgess Publishing Co., Minneapolis, Minn. 1949.

# BIOCHEMISTRY OF THE USTILAGINALES

## I. PRELIMINARY CULTURAL STUDIES OF *USTILAGO ZEAE*[1]

By R. H. Haskins[2]


### Abstract

A program of screening a wide range of fungi for ability to "ferment" glucose and various agricultural wastes and surpluses in submerged culture revealed that species of *Ustilago* were capable of rapid dissimilation of carbohydrates with the production of (1) a complex substance appearing as long needlelike optically active crystals in the culture mixture, (2) fats and sterols, and (3) a range of new antibiotic substances designated as Ustizeain A, B, etc. The yields of (1) and (2) are closely related and together they constitute the major portion of the by-products of metabolism of the smut. The antibiotics (3) are stable and effective *in vitro* against Gram-positive bacteria such as *Bacillus subtilis* and *Mycobacterium smegmatis*; against Gram-negative *Brucella bronchiseptica*; and against many fungi including species of *Aspergillus niger* and *A. flavus* groups, certain penicillia, *Verticillium*, and many other Fungi Imperfecti.


A great variety of complex reactions are carried out with ease by microorganisms, and since the majority of fungi have not yet been investigated for possible use to man, one of the projects in this new laboratory has been the screening of a wide range of fungi for ability to "ferment" glucose and various agricultural wastes and surpluses in submerged culture.

The term "fermentation" is used in the colloquial sense, meaning the formation of some product by a microbiological process. With most fungi the process is an oxidation, not a fermentation in the Pasteurian or scientific sense.

Early in this screening program it was found that, in shake cultures, *Ustilago zeae* (PRL-119) utilized over 87% of the total carbohydrate in a 5% glucose–nutrient solution in three days, producing elongated yeastlike budding cells sufficient in number to make the culture mixture creamy in color and consistency, and many long needlelike optically active crystals (Fig. 1). The cells themselves were heavily charged with oil globules. Furthermore the ease of isolation of the organism into pure culture and chance contamination of stored agar plates revealed strong antibiotic properties of substances produced by the organism.

An intensive program was initiated immediately to study the culture and physiology of the organism as well as to isolate and characterize the many metabolic by-products, which include the crystalline compound, various fats, sterols, and possibly alkaloids, and the antibiotic substances.

The present paper is the first in a series describing the results of these investigations. Later papers will describe comparative studies of the various strains, and biochemical studies of the crystalline substance, the fats, the sterols, the alkaloids, and the antibiotics.

[1] *Manuscript received December 9, 1949.*

*Contribution from the National Research Council Prairie Regional Laboratory, Saskatoon, Sask. Issued as paper No. 82 on the Industrial Utilization of Wastes and Surpluses and as N.R.C. No. 2095.*

[2] *Mycologist.*

## Methods

*Collection and Isolation of the Test Organisms*

The organisms used in these studies were isolated directly from infected plant material, from air contaminants, or from portions of mutating cultures. In the first method, portions of heavily smutted ears were shaken over, or broken up and streaked over, the surface of Difco potato dextrose agar or other suitable solid media. Germination of the chlamydospores was watched through the bottom of the plate, and when young colonies reached an appropriate size, those sufficiently isolated from other organisms were transferred to fresh plates. A few such transfers generally resulted in pure cultures. Though the present paper is concerned chiefly with *Ustilago zeae*, the organisms now in culture, together with source of material, are listed in Table I.

With *Ustilago zeae*, isolation was both rapid and easy, for the mere shaking of a portion of smutted cob above the surface of the agar resulted in innumerable small colonies of smut (Figs. 3, 4) with very few, much inhibited contaminating colonies. This inhibition of growth of contaminants was the first indication of the antibiotic ability of *U. zeae*.

Incubation of plates is normally at 28° to 30° C. for about a week, after which they are stored at room temperature. Cultures are being conserved successfully at 5° C. on short agar slants under sterile mineral oil following the technique described by Buell and Weston (1). Cultures conserved in this manner have retained their viability for over eight months, to date of writing.

*Cultural Technique*

Though earlier isolations were made successfully on potato dextrose agar, a search for better media was begun. In the preliminary screening tests the media were based on a mineral solution similar to that used by Whiffen (7) and known in this laboratory as MB-2. To this mineral base solution were added yeast extract, 0.06%; ammonium sulphate, 0.16%; calcium carbonate,

---

FIG. 1. *Dilution of culture mixture from shake flask of Ustilago zeae grown on mineral – yeast extract – urea – glucose medium for four days at 30° C. The long optically active needle-like crystals are easily distinguished from the small, short smut cells.* ×95 *approx.*

FIG. 2. *Fragments of optically active crystalline material taken directly from a thick layer on the walls of 9-liter glass 'fermentor' just above air–liquid interface.* ×166.

FIG. 3. *Surface of agar plate planted with finely divided fragments of heavily smutted corn kernels, after six days' incubation at 30° C. The wrinkled colonies are Ustilago zeae. Remarkably few fungous and bacterial colonies are present.* ×0.8 *approx.*

FIG. 4. *Surface of agar plate planted with spores shaken from heavily smutted corn cob, after four days' incubation at 30° C. The only two contaminants appearing on this plate were strongly inhibited. Colony "a" appears enlarged in Fig. 5.* ×0.7 *approx.*

FIG. 5. *Colony "a" (from Fig. 4). The solid black mass in center is the growth of Ustilago zeae. The material surrounding the colony in the agar is the optically-active crystalline metabolic by-product, which is in the form of characteristically grouped needlelike crystals.* ×7.

FIG. 6. *An early plate showing inhibition of Verticillium sp. by four day culture of Ustilago zeae.* ×0.6 *approx.*

PLATE I

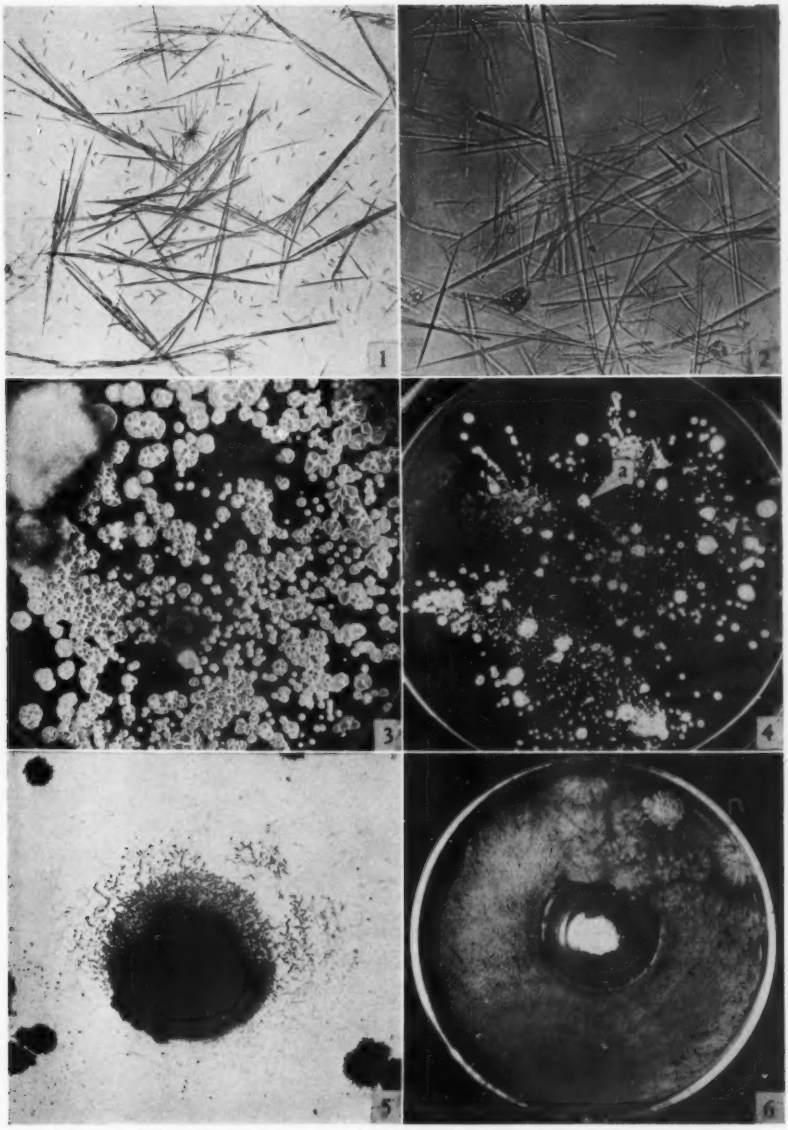

TABLE I

SPECIES AND STRAINS OF *Ustilago* PRESENTLY IN CULTURE, TOGETHER WITH SOURCE OF ORIGINAL MATERIAL

| PRL No. | Organism | Isolated from | Source of host material |
|---|---|---|---|
| 119 | *Ustilago zeae* | Corn (*Zea*) | G. A. Ledingham |
| 191 | *U. avenae* | ATC 10056 | A.T.C.C. |
| 199–200 | *U. zeae* | PRL-119 | R. H. Haskins |
| 201 | *U.* sp. | Air contaminant | A. C. Neish |
| 203–204 | *U. zeae* | PRL-119 | R. H. H. |
| 205 | *U.* sp. | Air contaminant | R. H. H. |
| 206 | *U. nigra* | Barley (*Hordeum*) | W. F. Hanna |
| 207–209 | *U. zeae* | PRL-119 | R. H. H. |
| 210, 212 | *U. tritici* | Wheat (*Triticum*) | W. F. H. |
| 211, 213 | *U. nigra* | Barley (*Hordeum*) | W. F. H. |
| 214 | *U. hordei* | Barley (*Hordeum*) | W. F. H. |
| 215, 221 | *U. avenae* | Oats (*Avena*) | W. F. H. |
| 216–218 | *U.* sp. | PRL 201 | R. H. H. |
| 219 | *U. nuda* | Barley (*Hordeum*) | W. F. H. |
| 220 | *U. kolleri* | Oats (*Avena*) | W. F. H. |
| 222-226 | *U.* sp. | Air contaminants | R. H. H. |
| 227, 228 | *U. zeae* | PRL 119 | R. H. H. |
| 316 | *U. nigra* | PRL 206 | R. H. H. |
| 317 | *U. zeae* | PRL 209 | R. H. H. |
| 373 | *U. neglecta* | *Chaetochloa lutescens* | I. L. Conners |

0.16%; and one carbon source such as glucose, 5%; filter paper, 1%; ground wheat straw, 5%; whole wheat meal, 5%; wheat germ, 5%; bran duster flour, 5%; whole corn meal, 5%; or whole rape seed meal, 5%. The yeast extract was usually omitted from whole grain meal and low grade flour media.

In later media the mineral base solution was greatly simplified following results of an experiment described elsewhere in this paper. This simplified mineral solution (MB-50) contained monobasic potassium phosphate, 0.1%; magnesium sulphate crystals, 0.04%; ferrous sulphate crystals, 0.003%; in distilled water. Corn steep liquor, 0.06%, and urea, 0.06%, are now used as sources of growth substances and nitrogen, and cerelose, fructose, or commercial sucrose as sources of carbon. Because of the high production of acid, the medium is normally buffered by the addition of about 0.15% calcium carbonate. The media are sterilized by autoclaving for 15 min. at 15 lb. When the volume of the medium is large it is more convenient to sterilize the calcium carbonate separately and add it under aseptic conditions to the sterilized medium.

Cultures are grown in 50 to 100 cc. amounts in 500 cc. cotton-plugged Erlenmeyer flasks or in 100 to 200 cc. amounts in 1000 cc. flasks, and shaken at about 200 r.p.m. on a rotary Gump shaker at 30° C. The fungus has been grown successfully in up to nine-liter glass, and 30-liter stainless steel fermentors.

Inoculation of flasks is by pipetting, under aseptic conditions, aliquots of suspensions of the organism in distilled water. These are normally prepared

by blending, with sterile distilled water in a sterile semi-micro Waring blendor, several entire colonies of the organism on agar. The smuts are very "variable" organisms, therefore it is essential to utilize several large colonies in order to prepare the physiologically homogeneous suspension necessary to ensure consistent results. After inoculation of flasks, control agar plates are inoculated from the last pipetteful as a check on contamination, and to provide inoculum for further experiments. In more recent work, inoculations of flasks are made directly from shake cultures, with extra flasks added to each run to ensure abundance of inoculum for succeeding experiments.

The blending technique has been used in all transfers from plates, whether to flasks or to plates, once large enough colonies have been obtained by streaking. When cultural studies revealed the excellence of urea as a nitrogen source, a mineral–urea–glucose agar (MUG 51, i.e. urea, 0.06%; yeast extract, 0.06%; glucose, 5%; agar, 1.5% in mineral base solution MB-50) has been used almost exclusively for maintaining smut cultures.

The pH determinations were made with a Beckman Model G pH meter and total carbohydrates were determined using Dreywood's anthrone reagent (2). Unless otherwise stated, the percentage of total carbohydrate utilized is based on total carbohydrate present before and after incubation, not considering evaporation, and is used as a general indication of total growth. In some cases of interest, percentage carbohydrate utilized was determined on the basis of soluble carbohydrate remaining in the medium.

## Experimental

The results obtained from the fermentation of the screening media by *Ustilago zeae* are worth reporting here (Table II). The crystals were present in great numbers in the glucose and the sucrose media where the pH of the culture mixture dropped appreciably in spite of the calcium carbonate present.

In the investigation of the dissimilation of glucose, it was found that the organism PRL-119, was able to break down 96% of the soluble carbohydrate in a 5% glucose – mineral base – yeast extract – ammonium sulphate medium, in less than four days when shaken at 30° C. with calcium carbonate as the buffer.

Using 5% sucrose as the carbon source and making the appropriate tests daily, it was found, as shown in Fig. 7, that growth was rapid, under the conditions of the experiment, reaching a maximum by the fourth day. The space between the soluble carbohydrate and the total carbohydrate curves indicates the amount of carbohydrate present as cell material plus crystals and other water-insoluble carbohydrate substances. The crystals appeared about the second day and increased in number until, on the fourth day, they were greater in number than the cells. By this time growth had ceased owing to practical completion of the "fermentation".

TABLE II

PERCENTAGE OF TOTAL CARBOHYDRATE IN VARIOUS MEDIA UTILIZED BY *Ustilago zeae* (PRL-119) GROWING IN FLASKS DURING THREE DAYS' SHAKING AT 30° C.

| Medium | pH | | Total carbohydrate utilized, % |
|---|---|---|---|
| | Start | Final | |
| Glucose 5% | 7.2 | 4.4 | 87.3 |
| Cellulose 1% | 8.2 | 8.2 | 7.7 |
| Wheat straw 5% | 6.7 | 8.5 | 76.2 |
| Wheat meal 5% | 6.6 | 7.6 | 25.5 |
| Corn meal 5% | 6.9 | 5.2 | 71.0 |
| Wheat germ 5% | 6.6 | 8.7 | 3.6 |
| Rape seed meal 5% | 6.3 | 6.3 | 4.8 |
| Sugar beet molasses 5% | 7.0 | 8.1 | 90.2 |
| Bran duster flour 5% | 6.6 | 6.6 | 0.0 |
| Rape seed cake 5% | 6.3 | 7.8 | 16.0 |
| Fir sawdust 5% | 6.1 | 6.7 | 4.2 |
| Table molasses 5% | 7.0 | 7.8 | 83.3 |
| Sucrose 5% | 7.0 | 4.9 | 85.4<br>99.5* |
| Dextrin 5% | 6.5 | 6.6 | 61.2 |
| Difco Penicillin | 5.6 | 8.6 | 2.1 |

* *Percentage based on soluble carbohydrate remaining in culture mixture, other values in column based on total carbohydrate in culture mixture.*

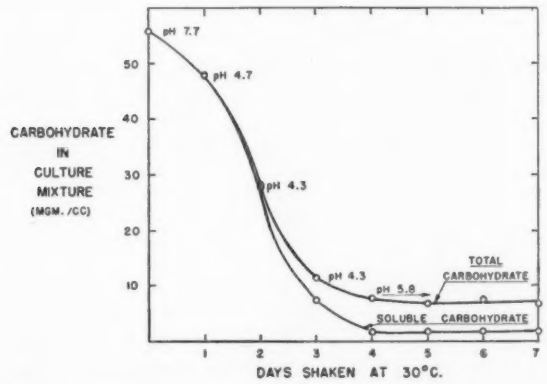


FIG. 7. *Fermentation of 5% sucrose by Ustilago zeae (PRL-119) expressed as mgm. per cc. carbohydrate in medium after various periods of growth.*

In the next series of cultures, the organism was grown on media of different sugar concentration. The results are shown in Fig. 8. With glucose in concentrations up to 5%, essentially all of the available sugar was fermented in the four days' incubation, though the production of insoluble materials (cells plus crystals) was particularly large in the 10% glucose culture.

In a comparison of the fermentation of various sugar sources in concentrations of 5, 10, and 15%, with each of the sugars, more acid was produced and less of the total carbohydrate dissimilated with the higher the concen-

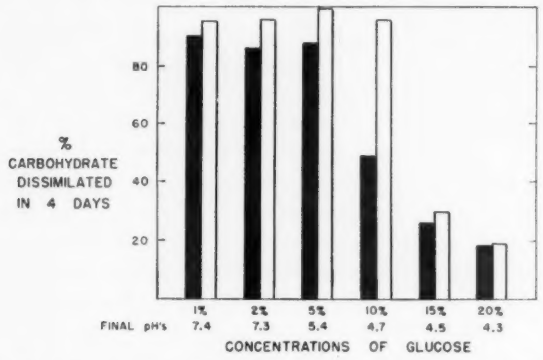


FIG. 8. *Fermentation of various concentrations of glucose by Ustilago zeae (PRL-119) when shaken for four days at 30° C. Solid columns determined from total carbohydrate in the culture mixture at end of incubation, broken columns determined from carbohydrate in solution.*

trations of sugar (Table III). Some cultures showed much oil in and around the individual cells. The total of solids produced was greatest with cerelose and least with light brown sugar.

TABLE III

"FERMENTATION" OF DIFFERENT CONCENTRATIONS OF VARIOUS SUGARS BY *U. zeae* (PRL-119)

| Sugar | Concentration, % | pH | | Carbohydrate dissimilated, % |
|---|---|---|---|---|
| | | Start | Final | |
| Cerelose | 5 | 7.2 | 6.7 | 88.7 |
| | 10 | 7.1 | 4.4 | 63.3 |
| | 15 | 6.9 | 4.4 | 44.5 |
| Commercial sucrose | 5 | 7.3 | 5 7 | 90.4 |
| | 10 | 7.3 | 4.6 | 61.7 |
| | 15 | 7.3 | 4.4 | 37.5 |
| Light brown sugar | 5 | 7.1 | 6.0 | 92.5 |
| | 10 | 7.0 | 4.7 | 38.3 |
| | 15 | 6.9 | 4.6 | 12.9 |
| Glucose c.p. | 5 | 7.2 | 5.8 | 88.1 |
| | 10 | 7.0 | 4.5 | 62.8 |
| | 15 | 7.0 | 4.4 | 33.6 |

Comparison of the fermentations of 5% glucose and of 5% sucrose by *Ustilago zeae* (PRL-119) with MB-2 and MB-50 as mineral bases, and urea and ammonium sulphate as nitrogen sources, showed that glucose is more

rapidly used than sucrose with MB-2 as the mineral base, but at the same rate with MB-50.

To determine the pH reached in unbuffered cultures, the mineral base–yeast extract–ammonium sulphate medium with 5% glucose, pH 6.02, was inoculated with a suspension of *U. zeae* (PRL-119) and shaken from three to seven days at 30° C. Each of the flasks at the end of its incubation period showed pH's ranging from pH 2.01 to 2.16. Growth of the fungus in these flasks was very sparse, with many thin-walled chlamydospore initials giving evidence of low pH or acid "damage".

Aeration is a very important factor in the growth of *Ustilago*. The results of a study of the effect of aeration on fermentation of glucose by *Ustilago zeae* (PRL-119) are shown in Table IV. With varying amounts of 5% glucose

TABLE IV

EFFECT OF AERATION ON DISSIMILATION OF GLUCOSE BY *Ustilago zeae* (PRL-119)

| Sugar concentration, % | Size of flask, cc. | Volume of contents, cc. | Flask plugged with: | Total carbohydrate dissimilated, % | Remarks |
|---|---|---|---|---|---|
| 5 | 1000 | 50 | Cotton | 91.6 | Medium size crystals |
| | | 100 | Cotton | 87.4 | Small crystals |
| | | 100 | Rubber stopper with small vent | 71.8 | No crystals |
| 5 | | 150 | Cotton | 90.4 | Long crystals |
| | | 200 | Cotton | 71.6 | Very long crystals |
| | | 250 | Cotton | 60.0 | |
| 5 | 500 | 100 | Cotton | 89.0 | Many medium crystals |
| 10 | | 50 | Cotton | 71.0 | |
| | | 100 | Cotton | 58.8 | |
| | | 50 | Stopper with small air vent | 51.8 | |
| | | 100 | | 31.4 | |
| | | 50 | Stopper with no vent | 22.0 | |
| | | 100 | | 28.0 | (Loose stopper) |

medium in liter flasks, inoculated and shaken for four days at 30° C. and using total carbohydrate remaining in the culture mixture as an indication of extent of fermentations, it was shown that decreased aeration due to increased quantities of medium in the flasks resulted in slower fermentations. Furthermore, decreasing aeration by reducing the size of air vent into the flask resulted in slower fermentation.

In a consideration of nitrogen sources, a number of cultures were set up using 5% glucose in a mineral base solution with one of several nitrogen sources. One series had yeast extract, the other had not. Buffering was with calcium carbonate and the cultures were shaken for four days at 30° C. At the end of the incubation period pH's were taken and residual total carbohydrate determined (Table V). It is interesting to note that the addition

TABLE V

FERMENTATION OF GLUCOSE BY *Ustilago zeae* (PRL-119) WITH VARIOUS NITROGEN SOURCES, WITH AND WITHOUT ADDED GROWTH SUBSTANCES DURING FOUR DAYS' SHAKING AT 30° C.

| Nitrogen source | | Yeast extract | pH | | Total carbohydrate utilized, % | Remarks |
|---|---|---|---|---|---|---|
| | | | Start | Final | | |
| $(NH_4)_2SO_4$ | 0.18% | Added | 6.46 | 5.41 | 90.0 | Few crystals |
| $KNO_3$ | 0.2% | Added | 6.71 | 5.51 | 90.3 | Crystals |
| | 0.2% | None | 6.69 | 5.32 | 91.0 | Crystals |
| Urea | 0.06% | Added | 7.33 | 5.78 | 87.4 | Many crystals |
| | 0.06% | None | 7.47 | 5.56 | 85.9 | Many crystals |
| $NH_4NO_3$ | 0.08% | Added | 7.65 | 4.76 | 90.9 | Crystals |
| | 0.08% | None | 7.88 | 4.73 | 74.1 | Crystals |

of growth substances in the form of yeast extract seemed to have but little effect on the fermentation. The various nitrogen sources seemed equally good though crystal production was much better with urea.

In order to simplify the mineral base used in making up the fermentation media, a series of flasks each carefully cleaned and containing 50 cc. of 5% sucrose in glass distilled water–mineral base medium were prepared so that to each a different one of the ions iron, sodium, potassium, phosphate, magnesium, sulphate, zinc, chloride, and calcium was **not** added. After inoculation and four days' shaking at 30° C., pH's were taken and total carbohydrate remaining in the culture mixture determined. The results were rather clear cut (Fig. 9). Cultures lacking in zinc, chloride, calcium, and iron grew well,

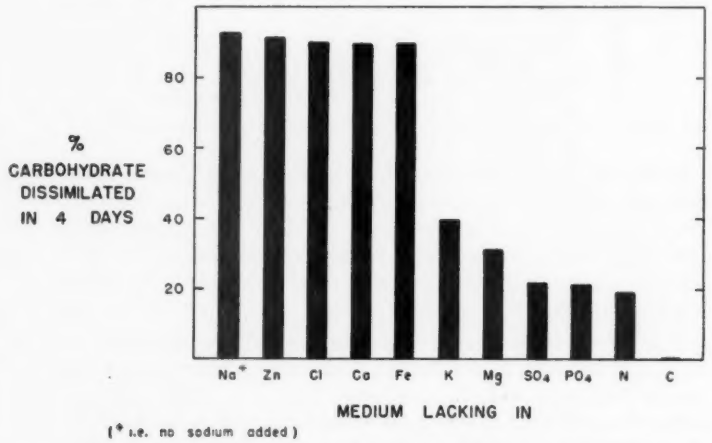


FIG. 9. *Ion and element requirements of Ustilago zeae (PRL-119) in dissimilation of glucose.*

dissimilating about 90% of the available carbohydrate, showing that these ions were either not essential for good growth of the organism or that they were present in sufficient quantities in the c.p. chemicals or from the pyrex glass to fulfill the needs of the plant. Ions such as potassium, magnesium, sulphate, and phosphate were obviously essential. It was on the basis of these results that the mineral base solution, MB-50, was designed.

A comparison of growth using MB-50 and using tap water as source of minerals showed that local tap water with added monobasic potassium phosphate, a nitrogen source, a carbon source, and a growth substance will **not** support adequately the growth of the fungus whereas MB-50 plus a nitrogen source and a carbon source will support excellent growth of the smut with or without added growth substances, though the presence of these does increase visibly the yield of by-products such as the crystals.

*Metabolic Products**

Much work has been done on the extraction and isolation of the metabolic by-products such as crystals, fats, sterols, and antibiotics. Reserving a detailed discussion for later papers, only general statements of results are given here.

The crystals formed in the culture medium (Figs. 1, 2, 5) have been recovered by treating the centrifuged culture solids with warm methanol, extracting the fats with ether, and allowing the substance to crystallize out from an aqueous solution of methanol under ether. The crystals are long and needlelike in the liquid culture medium and are soluble in methanol, warm ethanol, butanol, acetone, sodium hydroxide, sodium bicarbonate and insoluble in ethers, chloroform, carbon disulphide, amylacetate, water, etc. They are acidic, having reducing groups, and give a soapy solution with sodium hydroxide. Treatment of this soapy solution with hydrochloric acid results in a rubbery fruity odor and the precipitation, on occasion, of needlelike crystals, more usually, of an amorphous substance.

A series of fat fractions has been obtained from a single culture using different extracting procedures. Some of the fat mixtures were white and hard, others yellow and melting about 30° C. One mixture showed iodine numbers similar to that of lard, another similar to that of palm oil. About 50% of the fats are unsaponifiable, consisting mainly of sterols.

Experiments have shown that at least two comparatively stable, fat soluble antibiotic compounds, designated as Ustizeain A, Ustizeain B, etc. are produced by this organism. One is strongly active against Gram-positive bacteria including *Bacillus subtilis*, *Staphyllococcus aureus*, and *Mycobacterium smegmatis* and against Gram-negative *Brucella bronchiseptica*. The other is effective against certain penicillia, *Aspergillus* spp., *Verticillium* spp. (Fig. 6) and many other Fungi Imperfecti. Simultaneous inoculation of shake flasks with

** Applications have been filed for the patenting of the use of species of Ustilaginales, in particular of the genus Ustilago, for the production of the crystalline substance, the fats and steroids, and the antibiotics, together with methods for preparation and extraction of these substances.*

the smut and with heavy suspensions of *A. niger*, *B. subtilis*, etc. result in the growth of the smut only.

Agar plates inoculated in the center with one drop of a suspension of *Ustilago zeae* (PRL-119) and incubated at 30° C. for six days, and then flooded with a spore suspension of *Aspergillus niger* and incubated further, show complete inhibition of the *A. niger* in zones over 20 mm. from the edge of the smut colony. Furthermore, such plates kept in moist chambers at room temperature for periods of over eight weeks still showed no growth of the fungus in the clear zone, though it was well sown with spores from normal growth beyond the zone. This indicates that, in agar solution, the antibiotic substance is quite stable.

Extracts of the antibiotic substances have been made by adsorbing the active substance in the centrifugate on activated charcoal, eluting it with phosphoric acid, readsorbing on charcoal with subsequent elution by acid alcohol. A preferred method uses solvent extraction of the culture mixture with ether. The dried solids from extracts of cultures have retained antibiotic activity for over five months to date, though stored at room temperature.

## Discussion

Though much is known of the cytology, genetics, and phytopathology of species of the Ustilaginales, little work has been done on their physiology from other than a phytopathological aspect. The organisms, best known as the plant parasites causing the smut diseases of many grasses (e.g. *Ustilago zeae* causing common corn smut), are quite capable of growing in artificial culture on agar or in broth. The slow and restricted growth of the organisms on most stationary media (5) gives no indication of the greatly accelerated growth that takes place when liquid cultures are constantly shaken. Ninety-nine per cent of 5% sugar has been dissimilated by some strains in less than three days at 30° C. and a similar percentage of 10% sugar in four days at 30° C. with the production, in good yields, of a number of very interesting substances. These substances are being isolated, characterized in careful detail, and will be the subject of the subsequent papers in this series.

While some investigators of fungi capable of growing in a yeastlike manner have realized that large yields of unknown substances may be produced by the organism in artificial culture, the work on metabolic by-products, reported prior to the present paper, has been of a pharmacological nature. Impressed by the superficial similarity of the appearance on the host plant of smut diseases and the mycologically unrelated ergot (on rye), some early investigators surmised a pharmacological relationship. While there is no reason to suspect that any of the ergot principles are formed by smuts, it is interesting to note that the presence of an alkaloid, ustilagine (4, 6), has been reported and confirmed, and that extracts of corn smut (*Ustilago zeae*) have the power, like ergot, to cause contractions of uterine muscle, stimulate smooth muscle, increase intestinal tonus. Unlike ergot, however, smut extracts cause a fall

instead of a rise of blood pressure and no adrenal reversal. Cyanosis of coxcomb takes place, but it is a different type of blueing than that caused by ergot.

More recently, Mas (3) isolated from smutted corn the alkaloids ustilaginine and ustilagotoxine, which he states resemble ergotinine and ergotoxine respectively, and also demonstrated the presence of vitamin factor $D_2$. The present studies have already shown the presence of sterols, and it is quite possible that these may be produced in sufficient quantity to make irradiation and the production of vitamins quite feasible.

The use of the alkaloids from smutted corn as oxytocics, etc. was discontinued years ago, presumably because they were found not reliable. Growth of *Ustilago zeae*, in submerged culture on the large scale shown possible by the present work, may provide yields of uniform alkaloid products that may prove quite reliable and of considerable value.

Until work on the separation and identification of fats produced by *Ustilago zeae* in artificial culture has progressed further, nothing will be said on possible uses or value of the fats. The crystalline compound formed in the culture medium is proving to be a very complex molecule associated with the fats on the one hand and certain carbohydrates on the other.

The possible value of the formation of antibiotic compounds by an organism capable of using and of producing fatty substances, when these compounds are effective *in vitro* against such organisms as *Mycobacterium smegmatis* and *Brucella bronchiseptica*, can easily be appreciated. Toxicity tests of the antibiotic substances of the Ustizeain series await a more complete isolation and purification of the active materials, a work that is currently progressing rapidly, as is the work on the antifungal properties of the series.

## Acknowledgments

The author is indebted to Mr. I. L. Conners, and Drs. W. F. Hanna, A. C. Neish, and G. A. Ledingham for material from which certain of the strains used in these studies were isolated.

## References

1. Buell, C. B. and Weston, William H. Application of the mineral oil conservation method to maintaining collections of fungous cultures. Am. J. Botany, 34 : 555-561. 1947.
2. Dreywood, R. Qualitative test for carbohydrate material. Ind. Eng. Chem., Anal. Ed. 18 : 499. 1946.
3. Mas, Elena E. The alkaloids and vitamins of *Ustilago maydis* or *mappa*. Bol. soc. quím. Peru, 4 : 3-21. 1938.
4. Osol, A. and Farrar, G. E. *et al.* The dispensatory of the United States. 24th ed. J. B. Lippincott Co., Philadelphia. 1947.
5. Sartoris, G. B. Studies in the life history and physiology of certain smuts. Am. J. Botany, 11 : 617-647. 1924.
6. Testoni, P. Ricerche sull 'Ustilago Maidis. Arch. intern. pharmacodynamie, 44 : 278-288. 1933.
7. Whiffen, Alma J. Cellulose decomposition by the saprophytic chytrids. J. Elisha Mitchell Sci. Soc. 57 : 321-330. 1941.

## *Commonwealth Agricultural Bureaux*

This organization was set up by the Government of Canada in collaboration with the other Governments and responsible authorities of the British Commonwealth to provide up-to-date information in the form of journals, technical communications, and bibliographies on all aspects of science and practice as applied to agriculture, horticulture, and forestry.

JOURNALS. The following list comprises journals and other periodical publications. Subscription rates are quoted after each; in certain cases (journal marked with an asterisk) a 20 per cent deduction is made for subscribers in the British Commonwealth who send their subscriptions direct.

| | | | |
|---|---|---|---|
| Bulletin of Entomological Research | (40s.) | Dairy Science Abstracts* | (35s.) |
| Review of Applied Entomology (Series A) | (40s.) | Forestry Abstracts* | (45s.) |
| Review of Applied Entomology (Series B) | (20s.) | Forest Products and Utilization | (15s.) |
| Review of Applied Mycology* | (40s.) | Horticultural Abstracts* | (35s.) |
| Helminthological Abstracts | (35s.) | Field Crop Abstracts | (35s.) |
| Animal Breeding Abstracts* | (35s.) | Herbage Abstracts* | (35s.) |
| Veterinary Bulletin | (40s.) | Plant Breeding Abstracts* | (35s.) |
| Index Veterinarius | (100s.) | Soils and Fertilizers* | (35s.) |
| Nutrition Abstracts and Reviews | (63s.) | | |

### OCCASIONAL PUBLICATIONS

| | PRICE |
|---|---|
| List of Recorded Cotton Insects of the World. 1948. | 5s. |
| Report of the Fifth Commonwealth Entomological Conference, 22nd-30th July, 1948. | 7s. 6d. |
| West African Meliolineae. II. Meliolineae Collected by F. C. Deighton. 1948. | 12s. |
| The Plant Diseases and Fungi Recorded from Mauritius. 1948. | 5s. |
| Fungi of Barbados and the Windward Islands. 1948. | 3s. 9d. |
| A List of Plant Diseases of Economic Importance in Tanganyika Territory. 1949. | 3s. 9d. |
| List of Common Tropical Plant Diseases. 1947. | 3s. |
| Bibliography of Systematic Mycology. 1947. | 1s. 6d. |
| Seed Production of European Vegetables in the Tropics. 1948. | 2s. |
| Growth Substances and Their Practical Importance in Horticulture. 1948. | 12s. 6d. |
| Index to Horticultural Abstracts, volumes XI-XV, 1941-1945. 1949. | 35s. |
| Five Hundred Varieties of Herbage and Fodder Plants. 1948. | 15s. |
| The Technique of Breeding for Drought Resistance in Crops. 1948. | 2s. 6d. |
| New and Promising Varieties Recently Described in the Literature. 1948. | 2s. 6d. |
| The Practice of Soil Conservation in the British Colonial Empire. 1949. | 10s. |
| Bibliography of Soil Science, Fertilizers and General Agronomy, 1944-47. | 35s. |
| New Techniques for Mounting Fungi, 1949. | 1s. 6d. |
| Proceedings of the First Commonwealth Conference on Tropical and Sub-Tropical Soils, 1948. | 25s. |

All correspondence regarding above journals and other publications may be addressed in the first instance to:

COMMONWEALTH AGRICULTURAL BUREAUX,
CENTRAL SALES BRANCH,
PENGLAIS,
ABERYSTWYTH, GREAT BRITAIN.

# CANADIAN JOURNAL OF RESEARCH

## Notes on the Preparation of Copy

GENERAL:—Manuscripts should be typewritten, double spaced, and the **original and one extra copy** submitted. Style, arrangement, spelling, and abbreviations should conform to the usage of this Journal. Names of all simple compounds, rather than their formulae, should be used in the text. Greek letters or unusual sign should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts should be carefully checked before being submitted, to reduce the need for changes after the type has been set. If authors require changes to be made after the type is set, they will be charged for changes that are considered to be excessive. **All pages, whether text, figures, or tables, should be numbered.**

ABSTRACT:—**An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required.**

ILLUSTRATIONS:

(i) *Line Drawings:*—All lines should be of sufficient thickness to reproduce well. Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper **ruled in blue only**; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in **green, yellow, or red should not be used** unless it is desired to have all the co-ordinate lines show. Lettering and numerals should be neatly done in India ink preferably with a stencil (**do not use typewriting**) and be of such size that they will be legible and not less than one millimetre in height when reproduced in a cut three inches wide. All experimental points should be carefully drawn with instruments. Illustrations need not be more than two or three times the size of the desired reproduction, but the ratio of height to width should conform with that of the type page. **The original drawings and one set of small but clear photographic copies are to be submitted.**

(ii) *Photographs:*—Prints should be made on glossy paper, with strong contrasts; they should be trimmed to remove all extraneous material so that essential features only are shown. Photographs should be submitted **in duplicate**; if they are to be reproduced in groups, one set should be so arranged and mounted on cardboard with rubber cement; the duplicate set should be unmounted.

(iii) *General:*—**The author's name, title of paper, and figure number should be written in the lower left hand corner (outside the illustration proper) of the sheets on which the illustrations appear.** Captions should not be written on the illustrations, but typed on a separate page of the manuscript. All figures (including each figure of the plates) should be numbered consecutively from 1 up (arabic numerals). **Each figure should be referred to in the text.** If authors desire to alter a cut, they will be charged for the new cut.

TABLES:—Titles should be given for all tables, which should be numbered in Roman numerals. Column heads should be brief and textual matter in tables confined to a minimum. **Each table should be referred to in the text.**

REFERENCES:—**These should be listed alphabetically by authors' names, numbered in that order, and placed at the end of the paper.** The form of literature citation should be that used in the respective sections of this Journal. **Titles of papers should not be given in references listed in Sections A, B, E, and F, but must be given in references listed in Sections C and D.** The first page only of the references cited in papers appearing in Sections A, B, and E should be given. **All citations should be checked with the original articles.** Each citation should be referred to in the text by means of the key number; in Sections C and D the author's name and the date of publication may be included with the key number if desired.

The *Canadian Journal of Research* conforms in general with the practice outlined in the *Canadian Government Editorial Style Manual*, published by the Department of Public Printing and Stationery, Ottawa.

## Reprints

Fifty reprints of each paper without covers are supplied free. Additional reprints, if required, will be supplied according to a prescribed schedule of charges. On request, covers can be furnished at cost.